DEEP GENETIC DIFFERENTIATION IN THE LITTLE TUNNY FROM THE MEDITERRANEAN AND EAST ATLANTIC

J. Ollé[1], G. Hajjej[2], D. Macias[3], S. Saber[3], P. G. Lino[4], R. Muñoz-Lechuga[4], P. J. Pascual Alayón[5], D. Angueko[6], F. Ngom Sow[7], N'G. C. Diaha[8], F. Lucena Frédou[9], J. Viñas[1*]

SUMMARY

*In this study we present the analysis of the stock structure of Little tunny (*Euthynnus alletteratus*) using the mitochondrial control region as a genetic marker. More than 500 individuals distributed in six locations were analyzed. Samples were from three main regions: Mediterranean (Tunisia and Spain), and northeast Atlantic (Portugal and Senegal) and southeast Atlantic (Côte d'Ivoire and Gabon). Deep genetic differences were found between these locations. The phylogenetic tree showed a complete reciprocal monophyly association of the individuals in two main regions: Portugal, Spain and Tunisia; and Senegal, Côte d'Ivoire and Gabon. The level of genetic differentiation between these two areas is similar to the differences found between species of the genus Euthynnus. These results suggest a scenario of having two species of Little tunny in its Mediterranean-Atlantic distribution. Further evidence with other non-linked genetic markers along morphological and meristic data is necessary to fully confirm these two putative species. However, based on these results a separate management unit can be considered: i) Northeast Atlantic/Mediterranean (Portugal, Spain and Tunisia), and ii) the northeast/Southeast Atlantic (Senegal, Côte d'Ivoire and Gabon).*

RÉSUMÉ

*Dans cette étude, nous présentons l'analyse de la structure du stock de la thonine commune (*Euthynnus alletteratus*) en utilisant la zone mitochondriale de contrôle comme marqueur génétique. Plus de 500 spécimens répartis sur six sites ont été analysés. Les échantillons provenaient de trois régions principales : Méditerranée (Tunisie et UE-Espagne), Atlantique Nord-Est (Portugal et Sénégal) et Atlantique Sud-Est (Côte d'Ivoire et Gabon). De profondes différences génétiques ont été constatées entre ces endroits. L'arbre phylogénétique affichait une association monophylique réciproque complète des spécimens dans deux régions principales : l'UE-Portugal, l'UE-Espagne et la Tunisie ; et le Sénégal, la Côte d'Ivoire et le Gabon. Le niveau de différenciation génétique entre ces deux régions est similaire aux différences constatées entre les espèces du genre Euthynnus. Ces résultats suggèrent un scénario de présence de deux espèces de thonidés mineurs dans sa distribution méditerranéenne-atlantique. Des preuves supplémentaires avec d'autres marqueurs génétiques non liés ainsi que des données morphologiques et méristiques sont nécessaires pour confirmer pleinement ces deux espèces supposées. Toutefois, sur la base de ces résultats, une unité de gestion distincte peut être envisagée : i) Atlantique Nord-Est/Méditerranée (UE-Portugal, UE-Espagne et Tunisie) et ii) Atlantique Nord-Est/Sud-Est (Sénégal, Côte d'Ivoire et Gabon).*

[1]Laboratori Ictiologia Genètica, Departament de Biologia, Universitat de Girona. 17071, Girona, Spain.
*Corresponding author: jordi.vinas@udg.edu
[2]National Institute of Marine Science and Technology, Tunisie
[3]Instituto Español de Oceanografía, Centro Oceanográfico de Málaga, Spain
[4]Instituto Português do Mar e da Atmosfera, Portugal
[5]Instituto Español de Oceanografía, Centro Oceanográfico de Canarias, Spain.
[6]Direction General des Peches et de l'Aqualculture, Gabón.
[7]Centre De Recherches Oceanographiques de Dakar, Senegal
[8]Centrer of Oceanology Research,Côte d'Ivoire
[9]Departamento de Pesca e Aquilcutura – Universidad Federal Rural de Pernambuco, Brasil

RESUMEN

*En este estudio presentamos el análisis de la estructura del stock de la bacoreta (*Euthynnus alletteratus*) utilizando la región de control mitocondrial como marcador genético. Se analizaron más de 500 ejemplares distribuidos en seis localizaciones. Las muestras procedían de tres regiones principales: Mediterráneo (Túnez y España), Atlántico nororiental (Portugal y Senegal) y Atlántico suroriental (Côte d'Ivoire y Gabón). Se hallaron profundas diferencias genéticas entre las tres localizaciones: El árbol filogenético presentaba una asociación monofilética recíproca completa de los ejemplares en dos regiones principales: Portugal, España y Túnez; y Senegal, Côte d'Ivoire y Gabón. El nivel de diferenciación genética entre estas dos zonas es similar a las diferencias halladas entre especies del género Euthynnus. Estos resultados sugieren un escenario con dos especies de bacoreta en su distribución atlántica-mediterránea. Son necesarias más pruebas con otros marcadores genéticos no vinculados con datos morfológicos y neríticos para confirmar más estas dos especies putativas. Sin embargo, en base a estos resultados, puede considerarse una unidad de ordenación separada: i) Atlántico nororiental/Mediterráneo (Portugal, España y Túnez) y ii) Atlántico suroriental/nororiental (Senegal, Côte d'Ivoire y Gabón).*

KEYWORDS

Small tuna, Stock structure, Little tunny (LTA), Euthynnus alletteratus, *Northeast Atlantic, Southeast Atlantic, Mediterranean, population genetics*

1. Introduction

The Little tunny (LTA) (*Euthynnus alletteratus*) along with the Atlantic Bonito (BON) (*Sarda sarda*), and Wahoo (WAH) (*Acanthocybium solandri*), have been identified as key species in Small tuna working program in ICCAT to gather information for of growth, maturity and stock structure (Anon. 2019). Knowledge of these biological parameters are key to implement correct management strategies for the fisheries of these species.

One of the most useful and traditionally applied methods for inferring stock structure is based on population genetics (Ward 2000). This parameter is essential to infer the fishery stocks and finally to determine the correct measure of fishery management. For all the possible methodologies for inferring the population structure of the species, the use of mtDNA sequence variation is one of the most traditionally used in marine pelagic species. Although, nowadays other methods for inferring population structure such as microsatellites or Single Nucleotide Polymorphism (SNPs) are also widely used. The use of sequence variation of the Control Region (D-loop region) of mtDNA (mtDNA CR) is still extremely useful for inferring a preliminary assessment of the population's structure and a first step for subsequent application other methodologies with high power of resolution. One of the clear advantages of the mtDNA CR from other methods is that this methodology is already optimized for the majority of Scombridae species in (Allaya et al. 2015; Ollé et al. 2019; Viñas et al. 2010; Viñas et al. 2011). That implies that it could be applied to large number of samples with a relative low cost.

In this study, we have analyzed 504 individuals of little tunny from six locations distributed between the Mediterranean, northeast and southeast Atlantic (**Table 1**). The samples have been acquired due to the participation of two short terms ICCAT contracts under the Small Tuna Year Program (SMTYP). We can consider that this is probably the most exhaustive sampling of this species realized to date.

2. Material and methods

Up to 504 Little tunny distributed in six different locations distributed in the Mediterranean, northeast and southeast Atlantic were analyzed. Of these, in two locations two temporarily differentiated samples were included in the analysis. A sample of Black skipjack form Vietnam (*E. affinis*) was also included in the analysis as outgroup species reference (see **Table 1**).

Genetical variability of all individuals was assessed following the methodological procedure in Viñas et al. (2004). Briefly, once the samples arrived at the LIG-UdG total genomic DNA was isolated. Following extraction, DNA was resuspended in 100 µl of deionized water. We amplified approximately 450 base pairs (bp) of the first (left) domain of the mitochondrial control region (mtDNA CR) with the L-strand primer L15998 (5'-TAC CCC AAA CTC CCA AAg CTA-3'), in combination with the H-strand primer CSBDH (5'-TgA ATT Agg AAC CAg ATg CCA g-3'). Amplification was carried out in 12.5 µl reaction volumes using approximately 50 ng (0.5 µl) of the isolated DNA as the template. Each PCR reaction contained 1X Taq DNA polymerase buffer, 1.5–2 mM $MgCl_2$, 200 mM of each dNTP, 10 pmol of each primer, and 0.5 U Taq DNA polymerase. Thermal cycles involved an initial denaturing step of 5 min at 94°C, followed by 35 cycles of denaturing at 94°C for 45 s, annealing at 50°C for 45 s, and extension at 72°C for 1 min. Negative controls were included in all PCR runs to ascertain that no cross-contamination took place. Double-stranded DNA products were purified and subsequently were sequenced unidirectionally using the BigDye Kit v3.1 (Applied Biosystems) on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). When sequencing results were ambiguous, the amplicon was sequenced in both directions. Sequence alignments were inspected using the Geneious v.R7. Sequence Phylogenetic tree was constructed using the Neighbor joining (Saitou and Nei 1987) procedure with the kimura 2-distance (Kimura 1980) with a resampling of 1000 bootstrap pseudoreplicates to assess the robustness of the branches in the tree. Haplotype (h) (Nei and Tajima 1981) and nucleotide diversity (π) (Nei 1987) were estimated from haplotype frequencies and haplotype divergence based on a pairwise distance matrix in ARLEQUIN v. 3.5 (Excoffier and Lischer 2010). The geographical structure for each species was estimated using analysis of molecular variance (AMOVA) (Excoffier et al. 1992) based on the pairwise matrix of distances between haplotypes. The haplotypic correlation measure (Φ_{ST}) was estimated for all possible permutations among regions for each species. The significance level of each haplotypic correlation was tested by conducting a non-parametric permutation procedure 10,000 times in ARLEQUIN.

3. Results

Of the 399 nucleotide positions in the Little tunny mtDNA-CR alignment 148 were variable. In addition, only 160 (out of 504 individuals) distinct haplotypes were detected (**Table 1**). Thus, the overall haplotypic diversity was relatively low with some locations presenting an haplotypic diversity as low as $h = 0.643$ in the Portugal location. The nucleotide diversity was low in all locations (range from 0.003 to 0.011), but the overall diversity was relatively high (0.052). These discrepancies between the intra and inter-locations nucleotide diversity is consequence of the high divergence of two groups of sequences in Little tunny (**Figure 1**) with a net average between groups of $D_A = 0.084 \pm 0.014$. A further inspection of the phylogenetic tree reveals a complete association of individuals from different locations to the two groups of haplotypes detected in the tree (**Figure 1**). All sequences from individuals from Senegal, Côte d'Ivoire and Gabon, are grouped together, and separated from the locations of Portugal, Tunis and Spain. Correspondingly, there is a clear genetic differentiation between these two groups of locations (**Table 2**). In this analysis, a sample of *E. affinis* (Black skipjack) from Vietnam (VNM) was include in the analysis in order to assess if the degree of genetic differentiation is at species levels. It can be observed that the levels of Φ_{ST}s between *E. affinis* and the rest of locations is similar to the level of differentiation between the samplings from the groups of locations described before.

4. Discussion

Little tunny presents a surprising result of extremely deep genetic differentiation between the locations analyzed. A complete reciprocally monophyletic association of locations can be seen in the phylogenetic tree. Samples from the Mediterranean/Northeast Atlantic (Tunis, Spain and Portugal) are grouped together and clearly differentiated from the samples Senegal, Côte d'Ivoire and Gabon. The level of genetic differentiation between these two groups (average $\Phi_{ST} = 0.941 \pm 0.009$) is extremely similar that the level of genetic differentiation between two species of Euthynnus: Little tunny and Black skipjack (*E. affinis*) (average $\Phi_{ST} = 0.946 \pm 0.023$). Considering that this last comparison, Little tunny vs Black skipjack, can be accepted as a threshold for determining species in the Genus of *Euthynnus*, then the amount genetic differentiation observed between Mediterranean/Northeast Atlantic and East Tropical Atlantic can be considered at species level. The boundary of these two putative species could be somewhere between Senegal and the south of Portugal.

To fully confirm the presence of two differentiated species in the Little tunny distribution and considering the approach of genealogical species concept (Avise and Ball 1990), complementary data using nuclear genetic markers and morphically-meristic results are needed. What is still unknown is the boundary between these two areas. It can be anticipated that the region between Morocco and Mauritania could act as barrier between these two differentiated genetic pools, but further sampling in this area is essential to determine where the limit of gene flow is located.

Several pelagic species show some degree of genetic heterogeneity between north Atlantic and Tropical Atlantic (González et al. 2008; Martínez et al. 2006; Palko et al. 1981; Silva et al. 2014; Smith et al. 2015; Zarraonaindia et al. 2012), but to our knowledge none of the pelagic species studied in a similar zone present such deep of genetic differentiation. It is unknown what is the cause to produce this unexpected pattern of the distribution of genetic variability in the little tunny.

Finally, this study can be a starting point for stablishing correct fishery management measures by ICCAT for Little tunny. The results present here clearly challenges the ICCAT's management areas adopted for Little Tunny. Thus, at least two different genetic pools are present in the ICCAT area of NE-Atlantic/BILL94B. On the contrary, no genetic differentiation has been found between the location of Senegal (NE-Atlantic /BILL94B) and the two locations of SE-Atlantic/BIL 97 (Côte D'Ivoire and Gabon). Similarly, in the north, several locations that belong to different ICCAT areas: Portugal from NE-Atlantic /BILL94B, and Tunis and Spain form from MD/BIL95, share the same genetic pool.

5. Acknowledgements

This study was partially funded by the European Union (EU Grant Agreement No. SI2.796428 - Strengthening the scientific basis for decision-making in ICCAT).

References

Allaya H, Faleh AB, Hattour A, Trabelsi M, Viñas J. 2015. Disparate past demographic histories of three small Scombridae (Actinopterygii) species in Tunisian waters. Hydrobiologia. 758(1):19-30.

Anon. 2019. Report Of The 2019 Iccat Small Tunas species Group Intersessional Meeting. ICCAT Collective volume of scientific papers. 76(7):1-89.

Avise JC, Ball JRM. 1990. Principles of genealogical concordance in species concepts and biological taxonomy. Oxford Surveys in Evolutionary Biology. p. 45-67.

Excoffier L, Lischer HEL. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Res. 10(3):564-567.

Excoffier L, Smouse PE, Quattro JM. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics. 131(2):479-491.

González EG, Beerli P, Zardoya R. 2008. Genetic structuring and migration patterns of Atlantic bigeye tuna, *Thunnus obesus* (Lowe, 1839). BMC Evol Biol. 8:252.

Kimura M. 1980 A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16:111-120.

Martínez P, González EG, Castilho R, Zardoya R. 2006. Genetic diversity and historical demography of Atlantic bigeye tuna (*Thunnus obesus*). Mol Phylogen Evol. 39(2):404-416.

Nei M. 1987. Molecular evolutionary genetics. New York: Columbia University Press.

Nei M, Tajima F. 1981. DNA polymorphism detectable by restriction endonucleases. Genetics. 97(1):145-163.

Ollé J, Macías D, Saber S, José Gómez-Vives M, Pérez-Bielsa N, Viñas J. 2019. Genetic analysis reveals the presence of frigate tuna (Auxis thazard) in the bullet tuna (Auxis rochei) fishery of the Iberian Peninsula. Bull Mar Sci. 95(2):317-325.

Palko BJ, Beardsley GL, Richards WJ, United States. National Marine Fisheries S. 1981. Synopsis of the biology of the swordfish, Xiphias gladius Linnaeus. Seattle, Wash.: U.S. Dept. of Commerce, National Oceanic and Atmospheric Administration, National Marine Fisheries Service.

Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 4(4):406-425.

Silva G, P. Lima F, Martel P, Castilho R. 2014. Thermal adaptation and clinal mitochondrial DNA variation of European anchovy. Proceedings of the Royal Society B: Biological Sciences. 281(1792):20141093.

Smith BL, Lu C-P, Garcia-Cortes B, Vinas J, Yeh S-Y, Bremer JRA. 2015. Multilocus Bayesian Estimates of Intra-Oceanic Genetic Differentiation, Connectivity, and Admixture in Atlantic Swordfish (Xiphias gladius L.). PLoS One. 10(6).

Viñas J, Alvarado Bremer JR, Pla C. 2004. Phylogeography of the Atlantic bonito (*Sarda sarda*) in the northern Mediterranean: the combined effects of historical vicariance, population expansion, secondary invasion, and isolation by distance. Mol Phylogen Evol. 33(1):32-42.

Viñas J, Alvarado Bremer JR, Pla C. 2010. Phylogeography and phylogeny of the epineritic cosmopolitan bonitos of the genus *Sarda* (Cuvier): inferred patterns of intra- and inter-oceanic connectivity derived from nuclear and mitochondrial DNA data. J Biogeogr. 37(3):557-570.

Viñas J, Gordoa A, Fernández-Cebrián R, Pla C, Vahdet Ü, Araguas R. 2011. Facts and uncertainties about the genetic population structure of Atlantic bluefin tuna (*Thunnus thynnus*) in the Mediterranean. Implications for fishery management. Rev Fish Biol Fish. 21(3):527-541.

Ward RD. 2000. Genetics in fisheries management. Hydrobiologia. 420:191-201.

Zarraonaindia I, Iriondo M, Albaina A, Pardo MA, Manzano C, Grant WS, Irigoien X, Estonba A. 2012. Multiple SNP markers reveal fine-scale population and deep phylogeographic structure in European anchovy (Engraulis encrasicolus L.). PLoS One. p. e42201.

Table 1. Sampling description of little tunny and molecular diversity indices. Year, year of sampling. N, number of individuals; M, number of haplotypes; *h*, haplotypic diversity; π, nucleotide diversity.

Areas and locations	Code	Year	N	*M*	*h*±*SD*	π±*SD*
Mediterranean (MD/BIL95)						
Tunisia	TUN2018	2018	46	12	0.644 ± 0.077	0.005 ± 0.003
Spain	ESP2019	2018*	71	27	0.710 ± 0.060	0.004 ± 0.003
North East Atlantic (AT-NE/BIL94B)						
Portugal	PRT2018	2018	32	9	0.643 ± 0.094	0.003 ± 0.002
Portugal	PRT2019	2019*	90	24	0.633 ± 0.059	0.003 ± 0.002
Senegal	SEN2018	2018	50	31	0.958 ± 0.018	0.010 ± 0.006
Atlantic South East (AT-SE/BIL97)						
Côte d'Ivoire	CIV2018	2018	47	36	0.981 ± 0.010	0.011 ± 0.006
Côte d'Ivoire	CIV2019	2019*	100	55	0.972 ± 0.008	0.009 ± 0.005
Côte d'Ivoire (Spain)	ESP-CIV	2018*	23	20	0.984 ± 0.019	0.009 ± 0.005
Gabon	GAB2019	2018-2019*	45	33	0.981 ± 0.010	0.008 ± 0.005

Table 2. Pairwise genetic differentiation among Little tunny samples. Below diagonal, Φ_{ST}s values. Above diagonal, *P*-values. In bold, *P*-values significant after multiple testing. Samples code as Table 1. VNM, a sample from Vietnam (E. affinis) it is included in the analysis.

	PRT2018	TUN2018	PRT2019	ESP2019	SEN2018	CIV2018	CIV2019	ESP-CIV	GAB2019	VNM*
PRT2018	*	0.202	0.572	0.443	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
TUN2018	0.008	*	0.563	0.487	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
PRT2019	-0.004	-0.004	*	0.426	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
ESP2019	-0.001	-0.002	0.000	*	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
SEN2018	0.939	0.936	0.950	0.940	*	0.232	0.551	0.279	0.419	**0.000**
CIV2018	0.933	0.931	0.947	0.937	0.005	*	0.163	0.632	0.942	**0.000**
CIV2019	0.927	0.926	0.939	0.931	-0.002	0.005	*	0.454	0.170	**0.000**
ESP-CIV	0.949	0.943	0.958	0.947	0.005	-0.005	-0.001	*	0.438	**0.000**
GAB2019	0.946	0.942	0.955	0.945	0.000	-0.010	0.005	0.000	*	**0.000**
VNM	0.973	0.965	0.973	0.965	0.927	0.921	0.920	0.933	0.935	*

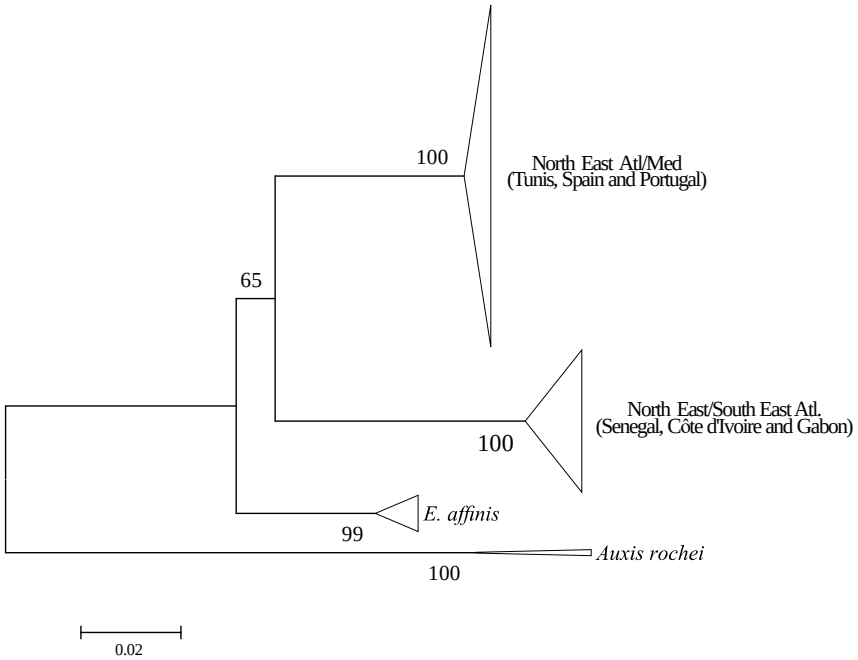

Figure 1. Rooted phylogenetic tree of the 160 Little tunny mtDNA-CR haplotypes. Tree is rooted with an outgroup (*Auxis rochei*). Black skipjack (*E. affinis*) from Vietnam were also included in the analysis. To facilitate visualization individuals from the respective areas described in Table 1 were collapsed together (grey triangles). Values in branches are bootstrap percentages above 65% consistency.