

MITOCHONDRIAL GENETIC CHARACTERIZATION OF BLUEFIN TUNA (*THUNNUS THYNNUS*) FROM THREE MEDITERRANEAN (LIBYA, MALTA, TUNISIA); AND ONE ATLANTIC LOCATIONS (GULF OF CADIZ)

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SUMMARY

None of the previous genetic analysis found significant differences within the Mediterranean Sea. However, a more exhaustive analysis is needed inside this sea. In this sense, we have analyzed the mitochondrial control region sequence variability of three Mediterranean locations: Libya ($n = 22$), Tunisia ($n = 23$) and Malta ($n = 12$). Additionally a sample from the Gulf of Cadiz ($n = 24$) in the East Atlantic Ocean was also included in the study. The AMOVA analysis revealed slightly ($\Phi_{st} = 0.013$) but significant genetic differences ($P < 0.000$) for the Malta location, probably due to its small sample size ($n = 12$). On the other hand, when this location is removed from the analysis, no differences were found among the three remaining locations ($\Phi_{st} = 0.005$; $P = 0.097$), or when they were compared with the previous genetic data from the Mediterranean ($\Phi_{st} = 0.002$; $P = 0.149$).

RÉSUMÉ

Aucune analyse génétique précédente n'a révélé de différences importantes en Méditerranée. Une analyse plus exhaustive est toutefois nécessaire dans cette mer. A cet effet, nous avons analysé la variabilité des séquences de la zone de contrôle mitochondriale de trois régions de la Méditerranée : la Libye ($n = 22$), la Tunisie ($n = 23$) et Malte ($n = 12$). Un échantillon provenant du Golfe de Cadix ($n = 24$) dans l'océan Atlantique est également été inclus dans l'étude. L'analyse AMOVA a révélé de légères ($\Phi_{st} = 0.013$) mais significatives ($P < 0.000$) différences génétiques pour la région de Malte, probablement en raison de sa petite taille d'échantillonnage ($n = 12$). Par ailleurs, lorsque l'on retire cette région de l'analyse, aucune différence n'apparaît entre les trois régions restantes ($\Phi_{st} = 0.005$; $P = 0.097$) ni quand on les compare avec les données génétiques précédentes de la Méditerranée ($\Phi_{st} = 0.002$; $P = 0.149$).

RESUMEN

Ninguno de los anteriores análisis genéticos encontró diferencias significativas en el Mediterráneo. Sin embargo, es necesario realizar un análisis más exhaustivo en dicho mar. En este sentido, hemos analizado la variabilidad de la secuencia de la región de control mitocondrial de tres localizaciones del Mediterráneo: Libia ($n = 22$), Túnez ($n = 23$) y Malta ($n = 12$). También se incluyó en el estudio una muestra del golfo de Cádiz ($n = 24$), en el océano Atlántico este. El análisis AMOVA revelaba diferencias genéticas ligeras ($\Phi_{st} = 0.013$), pero significativas ($P < 0.000$) para la localización de Malta, probablemente causadas por el pequeño tamaño de la muestra ($n = 12$). Por otro lado, cuando se elimina esta localización del análisis, no se encuentran diferencias entre las tres localizaciones restantes ($\Phi_{st} = 0.005$; $P = 0.09$), o cuando se comparan éstas con los datos genéticos anteriores del Mediterráneo ($\Phi_{st} = 0.002$; $P = 0.149$).

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KEY WORDS

Mitochondrial DNA, sequencing, genetic characterization, bluefin tuna, Mediterranean Sea, Atlantic Ocean.

1. INTRODUCTION

It is well accepted the presence of a single spawning area for the bluefin tuna in the Mediterranean Sea (Richards, 1976). This assumption has led to the acceptance of a single bluefin population within the Mediterranean Sea (ICCAT, 1997). Recently, several recent population genetics studies, using both nuclear (Pujolar, 2001) and mitochondrial (Viñas, 2001) molecular markers, could not reject the hypothesis of a single genetic population of the Mediterranean bluefin tuna. However, the opportunity of having samples from new localities, which never have been previously analyzed, could corroborate the single population supposition in the Mediterranean.

In this study we have analyzed three Mediterranean locations (Libya, Tunisia and Malta) and one East Atlantic location (Gulf of Cadiz), caught on the aim of COPEMED Project, using the mitochondrial control sequence variability as genetic marker. Additionally, we have compared this new data with our previous data from the rest of the Mediterranean.

2. MATERIAL AND METHODS

Bluefin samples used for mitochondrial DNA sequence analysis were collected in three Mediterranean locations, Libya ($n = 22$), Tunisia ($n = 23$) and Malta ($n = 12$), and one Atlantic location: Gulf of Cadiz ($n = 24$) (see **Table 1** for sampling details). D-loop sequence of each individual was obtained following the laboratory outlines described in Viñas (2001). The combination of primers used was: L15998-PRO primer which was complementary to the tRNA^{Pro} flanking D-loop fragment, with CSBDH primer (Alvarado Bremer *et al.*, 1995), corresponding to control sequence block (CSB) of the mitochondrial D-loop region. Sequences were read in ABI prism 310 Genetic analyzer available in the LIG (Laboratory of Ichthyology Genetics) at the University of Girona.

Sequences were edited by eye with the programs SEQ ED. (version 1.3) and Bioedit (version 5.0.9; Hall, 1999) and aligned using *Thunnus thynnus thynnus* sequences as reference (GenBank accession number X82653). Sequence variation was assessed estimating nucleotide diversity (π , Nei, 1987) and haplotypic diversity (h ; Nei & Tajima, 1981) using the Arlequin package (version 2.0; Schneider, 1997). Gene phylogenia was reconstructed using the Neighbor-Joining (NJ) algorithm (Saitou & Nei, 1987) on a matrix of gamma Tamura Nei ($\alpha = 0,27$) (Tamura, K. & M. Nei, 1993; Wakeley, J., 1993). A bootstrap test (Felsenstein, 1985) of 1000 replicates was carried out to check the strength of each branch of the tree. All the phylogenetic calculations were performed using the MEGA package (version 2.1; Kumar *et al.*, 2001).

We also examined the extent of population subdivision using an analysis of molecular variance (AMOVA, Excoffier *et al.*, 1992), available in the software package Arlequin. The significance level of the population subdivision was determined by a 3000 fold non-parametric permutation procedure also implemented in the Arlequin software.

3. RESULTS AND DISCUSSION

We have obtained 373 bp sequences of the 5' mitochondrial control region of each individual. According to Viñas (2001) this section corresponds to the first domain of this mitochondrial region. The comparison of the 81 sequences revealed 67 haplotypes, with frequencies 3 to one. The haplotypic diversity for the overall samples was estimated to $h = 0.993 \pm 0.003$. Haplotypic diversity of each sample (**Table 2**) is very similar than the estimated for the entire sample and also with the previously

calculated for 269 Mediterranean bluefin tunas ($h = 0,999 \pm 0,001$; Viñas, 2001). Among the 67 distinct haplotypes 62 variables sites were found, with 23 singletons and 39 parsimonious (**Figure 1**). Thus, the level of nucleotide diversity for the entire sample is $\pi = 0.042 \pm 0.007$, figure very similar with the previous results already obtained for the whole Mediterranean ($\pi = 0.044 \pm 0.021$).

The tree topology clustered the sequences in two highly divergent clades (**Figure 2**), named clade I and clade II, supported by 100% bootstrap values. The clade II is highly divergent from the clade I, with a great sequence similarity to the albacore (*Thunnus alalunga*). As described previously the presence of the clade II sequences is probably related to a mitochondrial introgression between several tuna species (Chow & Kishino, 1995; Viñas, 2001). The clade II is approximately 5% of the individuals (4 of 81) which is no significant different (χ^2 ; $P = 0.602 \pm .155$) from the frequency (6%) previously found by Viñas (2001) for this clade in the Mediterranean.

For the AMOVA analysis we decided to remove the clade II sequences from the analysis. The highly divergence of this clade probably would inflate artificially the nucleotide diversity of the samples. The overall comparison among the four samples resulted the rejection of the homogeneity, with a slight differentiation ($\Phi_{st} = 0.013$) but with a significant probability ($P < 0.000$). However, the pair wise comparison of the samples (Taula 3) reveals the genetic differentiation is only found in the Malta location. Moreover, when 269 sequences from six different Mediterranean locations were included in the analysis, the AMOVA analysis also indicated significant differences only for the Malta location. Since this is the first time that we have found genetic differentiation within the Mediterranean and considering the sample size ($n = 12$) if this location, we suggest that this heterogeneity is probably caused by its small sample size. Clearly, a more exhaustive analysis should be done with sample size increasing.

When the Malta location is not included in the analysis of the molecular variance, none of the remaining locations differ significantly ($\Phi_{st} = 0.005$; $P = 0.097$). Similarly, the three remaining samples (Libya, Cadiz and Tunisia) showed no genetic differentiation when they were compared to the pooled Mediterranean locations ($\Phi_{st} = 0,002$; $P = 0,149$). Thus, these results don't reject the hypothesis of homogeneity in the Mediterranean.

In summary, the AMOVA results demonstrate the low genetic differentiation with the gulf of Cadiz, Libya and Tunisia locations. Thus, these locations are genetically closed related to each other and also to the rest of Mediterranean. In the other hand, the genetic differentiation of the Malta is probably due a small sample size effect, but clearly additional analyses involving comparisons fishes from the same year from different Mediterranean locations would be desirable to corroborate these findings.

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Table 1. Samples used in this study

<i>Location</i>	<i>n</i>	<i>date</i>	<i>Average fork length (cm)</i>
Libya	22	6/1999	206.1
Malta	12	6/2002	137.3
Tunisia	23	5-6/2001	230.3
Gulf of Cadiz	24	5/2000	213.5

Table 2. Samples sizes, number of haplotypes and molecular diversity indices for each sample and for the entire data set.

<i>Location</i>	<i>n</i>	<i>Number of haplotypes</i>	<i>Molecular diversity indices</i>	
			<i>h</i>	π^*
Libya	22	19	0.985 ± 0.018	0.020 ± 0.011
Malta	12	9	0.954 ± 0.046	0.016 ± 0.009
Tunisia	23	20	0.985 ± 0.009	0.015 ± 0.008
Gulf of Cadiz	24	23	0.996 ± 0.014	0.019 ± 0.011
Total set	81	67	0.993 ± 0.003	0.020 ± 0.010

* Nucleotide diversity calculated after removal of introgression type haplotypes.

Table 3. AMOVA pair wise comparison among the four different samples and the previous data from the Mediterranean. Values above diagonal shows the Φ_{st} and values below its probability.

	<i>Libya</i>	<i>Malta</i>	<i>Tunisia</i>	<i>Cadiz</i>	<i>Mediterranean</i>
Libya	--	0.029	0.009	0.002	0.001
Malta	0.003*	--	0.029	0.023	0.026
Tunisia	0.042	0.003*	--	0.002	-0.002
Gulf of Cadiz	0.207	0.000*	0.214	--	0.001
Mediterranean	0.465	0.0034*	0.027	0.750	--

*significant probabilities after Bonferroni correction.

	111356666	6677788999	9011223344	4456677777	7788888899	9999990000	1122233455	5677778888	9900014455	6677
1	GGCACTTCTA	AGCTTTTAGA	TATTTACTTA	TATCACAATA	ACCTGTT-CG	TCTTGAAGCG	AAGCAATTCT	CTGACATCAC	AGAGCGTAAA	TGAA
2C.....T.....T.....	GT.A.-AA.....T
3C.....T.....T.....T.....A.-AT.....
4A.-AG.....
5C.....C.....A.-AA.....C.....
6G.....A.-AA.....A.....
7A.-A
8C.....CC.....	C.....T.A.-AC.....
10A.-A	C.....
15C.....A.-AA.....
16A.-A
20C.....T.....	G.....A.-A	C.....A.....
25C.....A.-AT.....C.....	C.....G.....TG
34C.....T.....	G.T.A.-A	C.....
40	AAG..ACT.T	GAA..C...G	..A.T..CT	.CCA.G.CCT	CT.CAC.TTA	.TAATT.TTA	.G...C...	.CA.A.C-..	T.....C...	C...
77C.....CC.....	C.....T.A.-AT.....C.....
85T.....A.-AG.....
91A.....A.....A.-AA.....
128A.-AA.G.....
149G.....A.-AC.....
150	G.....A.-AG.....A.....
153C.....A.-AT.....
176A.-AT.....T.....
200CC.....CA.-AT.....
201	?.G...C.....A.-AC.....C.....
202A.-AT.....T.....
203	???.T.CT.T	.AA..C.GA.	.C.ACT..CT	.CCGGA.T.T	CTT.A.CCTA	C..AC-.CTAG.....
204?A.....
205CC.....A.-AA.....
206C.....A.-AC.....C.....A.....
207	AAG.TACT.T	GAA..C...G	..A.T..CT	.CCA.A.CCT	CT.CAC.TTA	.T.ATT.CTA	.G...C...	.CA.A.-..	T.....C...	C...
208C.....C.....T.....	G.....A.-A	C.....A.....T.....
209A.....T.....G.....
210C.....A.-A?????
211C.....A.-A	C.....
212C.....TT.A.-AC.....
213A.-AT.....G
214C.....	GT.A.-A	C.....C.....
215?	G.....A.-AA.....C.....A.....
216C.....A.-AT.....A.....	G.....C.....G.....
217A.-AA.....G.....
218G.....C.....CC.....	C.....T.A.-A	C.....C.....
219C.....CC.....	C.....G.....T.A.-AC.....
220CC.....CA.-AT.....C.....
221A.-AC.....A.....
222	-----C.....A.-AT.....	G.....G.....
223C.....T.....	GT.A.-AG.....A.....T
224C.....CC.....	C.....T.AC.-AC.....
238A.-AT.....T.....
239C.....T.....	G.....A.-A	C.....AT.....A.....T.....
240	AAG..ACT.T	GAA..C...G	..A.T..CT	.CCA.G.CCT	CT.CAC.TTA	.TAATT.CTA	.G...C...	.CA.A.C-..	T.....C...	C...
241A.-AT.....C.....
242C.....T.A.-AA.....C.....
243C.....T.A.-AA.....
244A.-AA.....A.....
245T.A.-A
246A.....
247A.-AGG.....G.....
248G.....A.-AT.....
249G.....A.-AT.....A.....
250G.....A.-AT.....T.....
251G.....A.-AT.....T.AA.....G.....
252C.....T.....	G.....A.-A	C.....A.....AT.AA.G.....G.....
253C.....T.....	G.....A.-A	C.....A.....T.....
254G.....A.-AG.....T.....T.....
255G.....A.-AA.....T.....T.....
256G.....A.-AA.....TT.....T.....

Figure 1. Variable sites in the 67 haplotypes. Haplotypes numeration is according to Viñas (2001).



Figure 2. Neighbor-joining tree with Tamura-Nei distance ($\alpha = 0.27$) of the 67 bluefin tuna haplotypes. Numbers on the branches are bootstrap values (1000 replicates)