Report for the
SHORT-TERM CONTRACT FOR ICCAT SMTYP FOR THE BIOLOGICAL SAMPLES COLLECTION FOR GROWTH, MATURITY AND GENETICS STUDIES

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SUMMARY

The small tuna year program (SMTYP) has implemented a strategy for improving data about biological data: In particular data about growth, maturity and stock structure are still unreliable. These three parameters are key for implementing correct fishery management strategies. In particular, the Small Tuna Species Group decided to prioritize three species based on their economic importance and the lack of knowledge of their biology: Little tunny (LTA) (*Euthynnus alletteratus*), Atlantic Bonito (BON) (*Sarda sarda*) and Wahoo (WAH) (*Acanthocybium solandri*). Accordingly, ICCAT and University of Girona (UdG) and with the participation of up 12 Institutions representing 12 distinct CPCs signed a short-term contract in order to improve the biological knowledge. The two objectives were: i) Collect biological samples for estimating growth parameters, assessing the maturity and stock structure analysis (populations genetics) of three small tuna species (LTA, BON and WAH); and ii) Provide preliminary analysis of the stock structure for one of the three species. The final aim is to provide scientific base management and conservation measures for these species. In this document, we present the report of this contract.

KEYWORDS: Small tuna, Biological data, Stock identification, growth, reproduction, sampling, Little tunny (LTA) (Euthynnus alletteratus), Atlantic Bonito (BON) (Sarda sarda), Wahoo (WAH) (Acanthocybium solandri).

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Introduction

The small tuna year program (SMTYP) has implemented a strategy for improving data about catches data (Task I) and catch-effort and size data (Task II). Several calls of tenders focusing these two tasks have been released during the last years. However, for the majority of species included in the Small tuna working group, the biological data, in particular growth, maturity and stock structure are still uncertain. These three parameters are key for implementing correct fishery management strategies that ultimately will allow the preservation of stocks without compromising the viability of the natural populations. In particular, the 2017 Small Tuna Species Group decided to prioritize three species based on their economic importance and the lack of knowledge of their biology. These species are: Little tunny (LTA) (*Euthynnus alletteratus*) Atlantic Bonito (BON) (*Sarda sarda*) and Wahoo (WAH) (*Acanthocybium solandri*).

Accordingly, ICCAT and University of Girona (UdG) through its representative, the Laboratory of Ichthyology Genetics (LIG-UdG), signed a short-term contract in order to improve the biological knowledge of several species of small tuna with the final aim to provide scientific base management and conservation measures for these species.

The objectives of the contract were:

I. Collect biological samples for estimating growth parameters, assessing the maturity and stock structure analysis (populations genetics) of three small tuna species (LTA, BON and WAH)
II. Provide preliminary analysis of the stock structure for one of the three species.

Objective I. Collect biological samples

In the contract and following the recommendations of the SMTYP, a sampling scenario was detailed involving the maximum number of contracting parties.

See Tables 1 and 2 for a summary of the number of samples provided. A more detailed description of the data associated to the collected samples can be found in Annex 1.

Table 1. Details the number of samples to be provided according to the call of tenders and the number of samples actually provided (in bold). *EU-Spain total is distributed in three regions (BIL95, BIL94 and BIL97) with a total of samples to be provided of 370 and total of samples provided 380. **Mauritania included 44 Frigate tuna (total of samples provided 158)

		BON		LTA		WAH		Totals	
MU-SA region code	Institution	To be provided	**Provided**	To be provided	**Provided**	To be provided	**Provided**	To be provided	**Provided**
CPC EEZ									
MD; BIL95									
Tunisie	National Institute of Marine Science and Technology	113	**112**	97	**97**			210	**209**
Algerie	Centre National de Recherche du Développement de la Pêche et de l'Aquaculture, CNRDPA	109	**60**	80	**35**			189	**95**
EU-Spain	Instituto Español de Oceanografía	107	**108**	98*	**88**			370*	**196 (367)***
AT-NE; BIL94									
EU-Portugal	Instituto Português do Mar e da Atmosfera	98	**66**	56	**80**			154	**146**
EU-Spain	Instituto Español de Oceanografía					165	**161**	370*	**161 (367)***
Morocco	Laboratoire des Pêches (Dakhla)	116	**80**	72				188	**80**
Mauritania**	Laboratoire Evalutaion des Ressources Vivantes Aquatiques	123	**114**	96		196		415	**114 (158)****
Liberia	National Fisheries and Aquaculture Authority			73	**5**			73	**5**
Senegal	Centre De Recherches Oceanographiques de Dakar	118	**119**	109	**50**			227	**169**
AT-SE; BIL97									
EU-Spain	Instituto Español de Oceanografía			98*	**23**			370*	**23**
Côte d'Ivoire	Centrer of Oceanology Research	81	**83**	92	**81**	122	**90**	295	**254**
Gabon	Direction General des Peches et de l'Aqualculture	52		67	**69**		**21**	119	**90**
S. Tomé e Príncipe	Direcção das Pescas	87	**35**	77	**50**	163	**35**	327	**120**
AT-SW; BIL96									
Brazil	Universidade Federal Rural do Semiárido					171	**30**	171	**30**
TOTAL		1004	**777**	917	**578**	817	**336**	2738	**1692**

Table 2. Samples collected by the involved CPCs by species and type. N/A number of samples provided but information still missing. --, samples no provided.

	MU-SA region code	Stock structure	Growth		Reproduction
	CPC EEZ	Muscle	Spine	otoliths	Gonads
BON	**MD; BIL95**				
	Tunisie	112		112 (Head)	112
	Algerie	N/A	N/A	N/A	N/A
	EU-Spain	108	108	108	103
	AT-NE; BIL94				
	EU-Portugal	66	66 (Spines and Heads)		66
	Morocco	80		80 (Head)	40
	Mauritania	114	114	--	114
	Senegal	119	119	119	42
	AT-SE; BIL97				
	Côte d'Ivoire	83	52	49	73
	Gabon	--	--	--	--
	S. Tomé e Príncipe	35	35	--	35
LTA	**MD; BIL95**				
	Tunisie	97	97	--	97
	Algerie	N/A	N/A	N/A	N/A
	EU-Spain	88	88	80	88
	AT-NE; BIL94				
	EU-Portugal	80		80 (Head)	80
	Morocco	--	--	--	--
	Mauritania	--	--	--	--
	Liberia	N/A	N/A	N/A	N/A
	Senegal	50	50	50	30
	AT-SE; BIL97				
	EU-Spain	23	--	--	23
	Côte d'Ivoire	81	81	56	81
	Gabon	69	N/A	N/A	N/A
	S. Tomé e Príncipe	50	50	50	50
	AT-NE; BIL94				
WAH	EU-Spain	161	161	122 (Head)	49
	Mauritania	--	--	--	--
	AT-SE; BIL97				
	Côte d'Ivoire	90	90	90 (otolith head)	65
	Gabon	21			
	S. Tomé e Príncipe	35	35	--	35
	AT-SW; BIL96				
	Brazil	30	--	--	--

Although we only partially received the samples committed for the all CPCs (Table 1), we consider that this is an excellent starting point for continuing with this work line. For instance, and to our knowledge the samples already collected for Atlantic bonito consist probably one of the most exhaustive sampling in any species of small tuna, namely regarding their geographical coverage, and therefore providing an excellent opportunity to have better insight in the biology of this species. Nevertheless, we have to be conscious that the proposed level of sampling has not been accomplished and plans has to be revisited to accommodate the shortage of sampling during the first year of this project. Two main problems can be detected:

1. Low sampling accomplished specially for WAH. For this species, we were able to provide the only 40% of the sampling.
2. Some CPCs failed to give the information about the sampling at the timing for including the information into the report.

These two problems are being resolved, with some CPCs currently finishing the sampling.

Causes for the problems mentioned above include:

1. Short term for realizing the sampling (although the contract was extended until end of March 2019). This is the major problem of this project/contract. Having samples for all participants and for all analysis in relative short time was very problematic.
2. A number of problems related to payment procedures were raised in the course of the project, which the Consortium could not solve until the end of December.
3. The timing designed for sampling for some the species and in some regions was out of the fishery period.

Objective II. Preliminary analysis of the stock structure for one of the three species.

Since the sampling was not complete for any of the species, we decided to genetically analyze all the samples available at our lab before the end of January 2019. It should be mentioned that some of the members of the Consortium (i.e, Senegal) already sent all the samples, but they arrived too late which preclude the inclusion of their analysis and reporting prior to the drafting of this report.

Genetic methods

For all species, we followed the methodological procedure of analysis as described in Viñas et al. (2004): analysis of the mitochondrial control region (mtDNA-CR) sequence variability. Briefly, once the samples arrived at the LIG-UdG, total genomic DNA was isolated from each specimen. 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 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 sin 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.

Atlantic Bonito (BON)

Samples analyzed: 291 (about 23 failed individuals will be reanalyzed to increase the sampling size).

The discrepancy between the samples received and the samples analyzed is a common outcome. Some of the samples failed during the laboratory procedure. Nevertheless, we were able to analyze the 92% of the samples arrived at the LIG-UdG.

Results

Genetic variation in Atlantic bonito was relatively high with 106 variable sites out of 394bp of the alignment. We identified up to 175b distinct haplotype from 291 sequences (Table 3). Accordingly, in all locations, the haplotypic diversity was close to one, ranging from 0.975 to 0.992. Nucleotide diversity was also high compared to the ones observed in other species of *Sarda* (Viñas et al. 2010), but within the range observed within the Mediterranean Sea for the Atlantic bonito (Viñas et al. 2004). This high sequence variation diversity is probably consequence of the presence of two highly divergent groups of sequences (See Figure 1), previously described in Viñas et al. (2004), as Clade I and Clade II. Clear genetic differentiation was observed, with a highly significant overall value of Φ_{ST}= 0.146 (*P-value* = 0.000). Pairwise comparisons of genetic differentiation among locations (Table 4) revealed that the location of Côte D'Ivoire was significantly different form the rest of locations. In addition, the clade distribution among locations (Table 3) was highly heterogenous (χ^2 = 41.25; df = 4; *P-value* < 0.0001), being the African location of Côte d'Ivoire the one with lowest frequency of Clade II haplotypes with only 4% of the individuals. This last result reinforces the idea that Côte d'Ivoire is genetically differentiated location.

Table 3. Preliminary results regarding Atlantic bonito sampling and molecular diversity indices. N, number of individuals; M, number of haplotypes; *h*, haplotypic diversity; π, nucleotide diversity. Distribution of clades along locations according the phylogenetic tree in Figure 1; Black, Clade I; white, Clade II.

Location	N	M	*h*	π	Clade distribution		
					I	II	
ESP	91	66	0.987 ± 0.005	0.069 ± 0.034	44	47	
PRT	61	44	0.975 ± 0.011	0.068 ± 0.034	31	30	
CIV	50	43	0.992 ± 0.006	0.018 ± 0.009	48	2	
MOR	40	32	0.982 ± 0.012	0.048 ± 0.024	32	8	
TUN	49	34	0.982 ± 0.008	0.066 ± 0.033	28	21	
ALL	291	175	0.987 ± 0.002	0.064 ± 0.031	183	108	

Table 4. Pairwise genetic differentiation among Atlantic bonito samples. Below diagonal, Φ_{ST}s values. Above diagonal, *P*-values. In bold, *P*-values significant after multiple testing.

Location	ESP	PRT	CIV	MAR	TUN
ESP		0.048	**0.000**	0.000	0.026
PRT	0.024		**0.000**	0.007	0.398
CIV	0.349	0.322		**0.004**	**0.000**
MOR	0.156	0.105	0.090		0.032
TUN	0.042	-0.003	0.267	0.054	

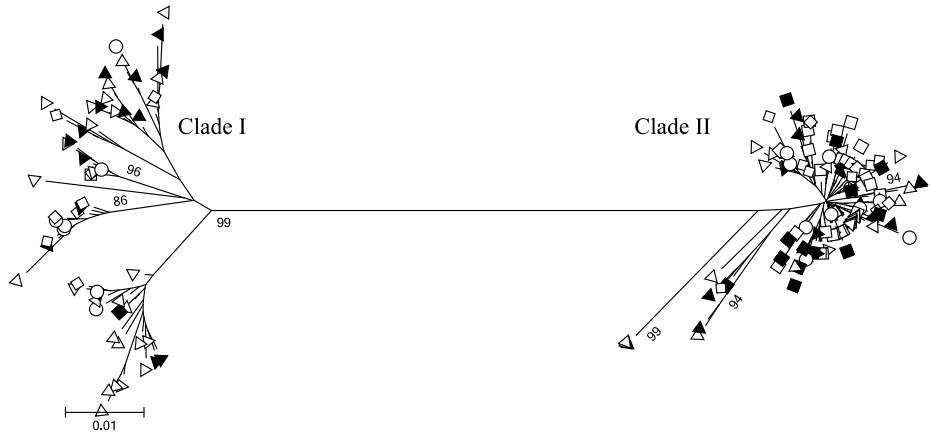

Figure 1. Unrooted phylogenetic tree of the 175 Atlantic bonito mtDNA-CR haplotypes.

Little tunny (LTA)

Samples analyzed: 118.

The discrepancy between the samples received and the samples analyzed can be attributed to technical errors during the laboratory procedure. In addition, we were able to detect at least 2 *Auxis thazard* samples in the Little tunny fishery, consequently, these individuals were not included in the analysis. Nevertheless, we were able to analyze the 88% of the samples arrived at the LIG-UdG.

Results

Genetic variation in Little tunny was relatively low compared to one observed for Atlantic bonito. Of the 406 nucleotide positions in the mtDNA-CR alignment 76 were variable. In addition, only 55 (out of 155 individuals) distinct haplotypes were detected (Table 5). Thus, the overall haplotypic diversity was relatively low with some locations presenting an haplotypic diversity as low as $h = 0.643$ in Portugal. The nucleotide diversity was low in all locations (range from 0.001 to 0.005), 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 2) 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 2). All sequences from individuals from Côte d'Ivoire are grouped together, and separated from the ones of Portugal and Tunis. Correspondingly, there is a clear genetic

differentiation between Côte D'Ivoire location from the remaining locations (Table 6). The high degree of genetic divergence between the African location from the two other locations together with the monophyletic group on Côte d'Ivoire observed in the phylogenetic tree suggests a possible scenario of speciation for Little tunny.

Table 5. Description of the Little tunny sampling and molecular diversity indices. N, number of individuals; M, number of haplotypes; *h*, haplotypic diversity; π, nucleotide diversity.

Location	N	M	*h*	π
PRT	32	9	0.643 ± 0.094	0.003 ± 0.002
CIV	44	36	0.987 ± 0.009	0.001 ± 0.005
TUN	42	15	0.737 ± 0.069	0.005 ± 0.003
ALL	118	55	0.879 ± 0.028	0.052 ± 0.026

Table 6. 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.

Location	PRT	CIV	TUN
PRT		**0.000**	0.139
CIV	0.936		**0.000**
TUN	0.011	0.928	

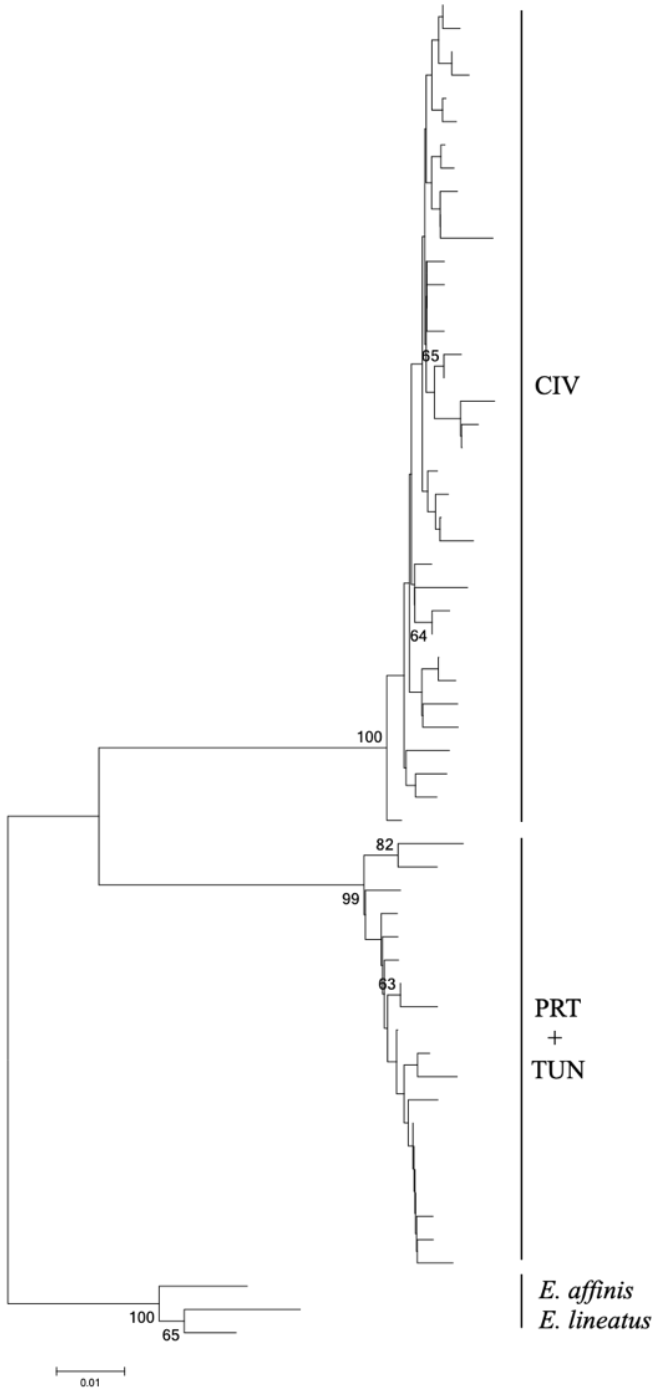

Figure 2. Rooted phylogenetic tree of the Little tunny mtDNA-CR haplotypes. This tree is rooted with representative mtDNA-CR sequences of *E. affinis* and *E. lineatus.* Number on the branches represents bootstrap levels above 60%.

Wahoo (WAH)

Samples analyzed: 26. All samples were from the Côte D'Ivoire location. No more samples were available.

Two individuals failed. we were able to analyze the 93% of the samples arrived at the LIG-UdG.

Results

The analysis of sequence of variation in Wahoo revealed up to 115 variable sites in the 453 nucleotides of the alignment of the mtDNA-CR. All sequences represented a different haplotype, and thus the haplotypic diversity was the maximum (h = 1.000 ± 0.011) (Table 7). In addition, the nucleotide diversity was the highest in all three species, probably related to the presence of two distinct groups of sequences in the phylogenetic tree (Figure 3). No further analyses were realized. Having a single location hampered our analysis considering the hypothesis of differential population structure in the area.

Table 7. Description of the Wahoo sampling and molecular diversity indices. N, number of individuals; M, number of haplotypes; h, haplotypic diversity; π, nucleotide diversity.

Location	N	M	h	π
CIV	26	26	1.000 ± 0.011	0.078 ± 0.039

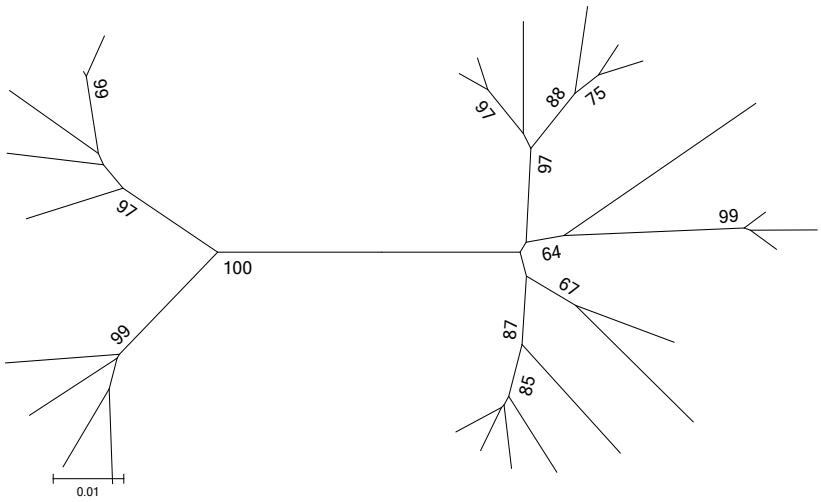

Figure 3. Unrooted phylogenetic tree of the Wahoo mtDNA-CR haplotypes. Number on the branches represents bootstrap levels above 60%.

General comments of the stock structure analysis

We were able to analyze about 650 individuals of BON, LTA and WAH. This included more than 150 individuals from Senegal of three species that we have not yet included in the preliminary results, as the genetic analysis is underway. From the two species that we were able to analysis the population structure (BON and LTA), we can conclude that specimens caught off Côte d'Ivoire are clearly genetically differentiated from the rest, with a surprising result for Little tunny, with a degree of differentiation at species level.

These results suggest that there are probably two different populations of BON and LTA in the east Atlantic. According to genetic stock structure analysis, for these two species, it is clear that the area AT-SE (BLI97) is genetically differentiated from the rest of the areas, suggesting a distinctive stock of the area AT-SE (BIL97) for these two species. A more detailed analysis, in particular adding samples from the boundary between the AT-NE (BIL94B) and AT-SE (BIL97), could help to determine the limits of the stocks. In this regard, the samples from Senegal (located at AT-NE; BIL94B) are very promising. As mentioned before, these samples are already in LIG-UdG and are currently being analyzed. Furthermore, we could infer that similar population structure may be found for other small tuna species with similar behavior. However, a more extensive sampling along the African coast is needed to confirm the boundaries between stocks.

General comments to the project

This contract has been an extremely ambitious program: three species with up to 13 institutions involved. The organization and execution have implied an extraordinary effort for all partners. We can congratulate to all participants in this project. However, we have to be aware that the sampling has not been completely accomplished (see details in the sampling description), which was one of the main objectives of the project. The deficiency in the sampling can be attributed to various causes, probably being the most important the low timing of execution and probably a too ambitious proposal.

Nevertheless, and besides these difficulties it should be mentioned that we are having one of the best an exhaustive sampling for any species of small tuna realized to date. Some of the results in stock structure, though preliminary, are extremely promising. We detected clear differentiated stocks structures in two species that can have a great impact in the management of these fisheries.

Aiming the continuity of the project, several improvements shall be taken in to consideration aiming the achievement of the proposed objectives:

1.- Multiyear program. Minimum two years. With this timeframe is possible to gather all the information needed for a one-year cycle of the species.
2.- Better communication between partners. We have profusely contact to all partners but some improvements in this matter should clearly be done
3.- Better turnover of information. For some samples and in some locations some of the information is not completed.

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References

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.

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

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.

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

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.