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FINAL REPORT OF THE SHORT-TERM CONTRACT FOR ICCAT SMTYP FOR THE BIOLOGICAL SAMPLES COLLECTION FOR GROWTH, MATURITY AND GENETICS STUDIES – Year #3

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SUMMARY

This document is the final report of the third year of the short-term contract of the Small Tuna Year Program by ICCAT, with the objectives of: a) conduct additional sampling aiming to fill the specific gaps of the biological samples for estimating the growth and maturity parameters for BON and LTA; b) estimate the referred parameters for both species, and preliminary provide preliminary results for WAH; and, c) refine the sampling and stock structure analysis for BON, LTA and WAH. A total of 374 individuals were collected: 145 of BON, 139 of LTA and 90 WAH. Initial target size class was accomplished only for BON in the Mediterranean. Small individuals are need in the Northeast and no samples were obtained in Southeast Atlantic. For LTA, total target sizes were not completely achieved in any case. However, preliminary results were obtained for growth and reproductive parameters. For BON, with samples arrived from Morocco, no genetic differentiation was detected, and the hypothesis provided in the previous contract is maintained. The population genetic analysis of WAH presents a scenario of homogeneous distribution.

KEYWORDS: Small tunas, Little tunny, Euthynnus alletteratus, Atlantic bonito, Sarda sarda, Wahoo, Acanthocybium solandri, growth, maturity, stock structure, genetics

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1.Introduction

The ICCAT Small Tunas Year Program (SMTYP) was adopted by the Commission in its 2012 meeting in Agadir (Morocco). The main objectives of this project are to improve historical Task I and II data and to collect biological data for small tunas (SMT), especially the growth, the maturity and stock structure data which are necessary for their assessment in the near future and thus provide scientific advice to ICCAT for their management.

The 2017 Small Tuna Species Group intersessional meeting decided to prioritize three species: little tunny (LTA) (*Euthynnus alletteratus*), Atlantic bonito (BON) (*Sarda sarda*) and wahoo (WAH) (*Acanthocybium solandri*), based on their economic importance and the lack of knowledge on their biology. As approved by the SCRS in 2017, the SMTYP collected biological samples aiming at describing the growth, maturity and stock structure on these three small tunas species in 2018 and 2019. In 2019, results on stock structure of two of the three species (BON and LTA) were provided and samples for growth and maturity were considered mostly satisfactory for the areas and species.

The main objective of the 2020 contract was: a) to conduct additional sampling aiming to fill the specific gaps of the biological samples for estimating the growth and maturity parameters for BON and LTA in the Atlantic and the Mediterranean Sea; b) estimate the referred parameters for both species, and preliminary provide preliminary results for WAH; and, c) refine the sampling and stock structure analysis for BON, LTA and WAH.

2.Results

In this report, we present the results for each objective.

2.1. Objective A. Collect biological samples

For the objective A, a total of nine CPCs were involved (Tunisie, EU-Spain, EU-Portugal, Morocco, Senegal, Côte d'Ivoire, Gabon, Brazil and EU-Malta). Sampling priority was given to fill specific gaps necessary to obtain the growth and maturity parameters for LTA and BON from geographical areas that the Small Tunas Species Group identified as of high priority (see **Table 1**), considering data collected in the last 2 years. Sampling of WAH from AT-SW was required for stock structure studies and also data from Mauritania, in order to validate BON stock boundaries, as pointed in the last years of the project. For both cases, no size target was requested. Also, during this contract, the complete data base (2018 -2021) was checked, cleaned and corrected in other to allow for an appropriate data analysis without bias.

A total of 374 individuals were collected during the present contract: 145 of BON, 139 of LTA and 90 WAH (**Table 2**). Note that not all the specimens collected reached in time the areas coordinators giving the enormous difficulties related to the pandemic (suspension of fishery activity, laboratories closed and shipping delays). Hence, the results presented by the areas coordinators included, so far, data arrived up to May. Analysis of others samples collected or lately arrived will be incorporated in the next contract. Moreover, the inherent difficulty of getting extreme size classes were also noted by the group.

Initial target size classes were only accomplished for BON in the Mediterranean. Small individuals are need in the Northeast and no samples were obtained in Southeast Atlantic (**Table 3, Figure 1**). For this species, specific gaps and hence bias in growth and reproduction parameters were noted, given the absence of the extreme sizes (see the growth and reproduction sections). For LTA, total target sizes were not completely achieved in any case. Nonetheless, preliminary results were obtained for growth parameters (see growth section) and gaps were identified to be fulfilled within the next contract. The excel file with the detailed information for species, region and sampling available by request to the secretariat.

2.2. Objective B. Estimate growth and reproduction for BON and LTA and provide preliminary results for WAH

2.2.1. Growth parameters (BON and LTA)

To date, Atlantic bonito and little tunny samples were collected by fishery observers in commercial fishing vessels and fish auctions from IPMA (Portugal), INRH (Morocco), INSTM (Tunisia), CRODT (Senegal), CRO (Cotê d'Ivoire), DGPA (Gabon), AquaBioTech (Malta), and IEO (Spain) over the Atlantic and Mediterranean (**Figure 2**). Specimens were measured for straight fork length (SFL, cm), location, sex, maturity stage and other biological parameters. For age and growth, samples consist of the first spine of the first dorsal fin or the first spine of the first dorsal fin plus otoliths, for comparison between structures. Other samples, such as tissue and gonads were also collected, when possible, for the other components of the biological study.

Due to difficulties in sample collection during 2020, a reduced number of BON samples were collected and received for processing. Since it was not possible to achieve the target of 250 spines for BON the Age and Growth coordinator considered that it would be the best option to increase the statistical robustness of the analysis by increasing the number of the spines per size class for LTA while maintaining the agreed budget. As a result, a total of 167 BON spines and 333 LTA spines were processed for age reading.

Sectioning of spines was performed at IPMA. Preparation of spines followed an adapted method of Ortiz de Zárate et al. (2007). The first dorsal fin spine was embedded individually in polyester resin for sectioning, three sections of approximately 0.5 mm were made at one and half distance (1.5 CW), one distance (1 CW) and at half distance of the condyle width (0.5 CW) (**Figure 3**). Spines were sectioned with an isomet low-speed cutting machine, using two pro slicer diamond blades in parallel. Sections were mounted on glass slides with a mounting medium to fix the sections permanently and properly labeled.

A total of 792 spine samples of little tunny (LTA) and 682 spines samples of Atlantic bonito (BON) have been collected to date for this study from the Northeast, Southeast Atlantic and Mediterranean Sea. A summary of the length range for each collected structure is presented in: little tunny- **Figure 4**; Atlantic bonito – **Figure 5**. Of these, 159 spines of little tunny and 130 spines of Atlantic bonito have already been sectioned. All slides were photographed under a dissecting microscope with a digital camera (**Figure 6**). The rest of the selected spines have already been cleaned, embedded in blocks or cut into sections.

For the little tunny species, 159 spines were processed and one was discarded due to poor visualization. Three cuts were made for each spine, analyzing a total of 474 sections (**Figure 7**). Three sections were compared with each other by observing the distance at which the rings are best observed. For this, various patterns were carried out that included the reading of annulus, section diameters, vascularization, loss of annulus due to vascularization, distance of annulus formation and difference in distances between *annulus*. The best distance from these results was the 1.0 CW, which will be selected to carry out the subsequent sections analyzes with the rest of the spines.

For the Atlantic bonito, 130 spines were processed and 14 were discarded due to poor visualization. Three cuts were made for each spine, analyzing a total of 348 sections (**Figure 8**). Three sections were compared with each other by observing the distance at which the rings are best observed. For this, various patterns were carried out that included the reading of annulus, section diameters, vascularization, loss of annulus due to vascularization, distance of annulus formation and difference in distances between *annulus*. The best distances from these results were the 0.5 CW and 1.0 CW. It is necessary increase the number of samples and size range to select the best section distance.

A preliminary analysis of the relationship between section spine diameter (mm) and fish size (FL - cm) (**Figure 9**), showed that area effect (North-east Atlantic + Mediterranean and South-east Atlantic) is significant for little tunny (P-value < 0.001). Not differences were observed between areas for Atlantic bonito.

At this stage no preliminary growth models were fit by area due to the low number of processed samples, particularly considering models have to be stock-specific, growth models would most likely converge at unreasonable results and with high uncertainty. In this case all samples were grouped to produce growth models (**Figure 10**).

Processing of otoliths of bonito and little tunny was outsourced to a specialized laboratory (Fish Ageing Services Pty Ltd in Australia). A total of 316 otolith samples were sent for analysis (255 for annual [120 Little tunny and 135 Atlantic bonito] and 61 for daily rings [43 Little tunny and 18 Atlantic bonito]).

According to the consortium specifications, sections of the otoliths will be obtained, for both annual and daily rings readings. Otoliths prepared for daily rings readings will be ground down using sandpaper of different grits until a section of approximately 50-80 μm is obtained that contains the primordium. Samples of small individuals are used for daily growth. Otoliths prepared for annual rings readings follow the same procedure as otoliths prepared for daily readings, but the final section thickness will be of about 250-300μm.

Small tunas have been the subject of several biological studies on age and growth worldwide and most studies have conducted age readings using the first dorsal fin spine. However, not all have used the same reproducible and comparable standardized method between studies.

It should be noted that no direct validation studies of growth band pair deposition exist for both species. Direct validation of band pair deposition can also be hindered by reabsorption or remobilization of bone in small tuna spines and therefore ideally it would be analyzed in otoliths. This is a major gap for ageing studies in small tuna species and future research should be focused on validating the band pair deposition. Validation would possibly involve oxy-tetracycline tagging (mark-recapture) and/or bomb radiocarbon studies.

2.2.2. Growth parameters (WAH)

For the Southwest Atlantic, with samples obtained specifically in Brazil, in the years of 2020 and 2021 a total of 90 heads of *Acanthocybium solandri* were collected for the extraction of *sagittae* otoliths, which will be used for shape analysis, microchemistry, as well for age and growth studies. During the previous contract, 38 heads were collected, totaling 128 pairs of collected otoliths, overtaking the initial goal of 100 samples. All information about the size, weight, and date of collection were stored in a Microsoft Excel spreadsheet, and the preliminary results are showed below. For this region, individuals measured from 68 to 177 (mean = 121.2) of fork length (**Figure 11**).

A total of 67 whole otoliths have been measured by a caliper, weighed on a precision scale, and then photographed using a reference scale, according to the protocol established in previous studies, using a stereo microscope with an image capture system for further shape analysis (**Figure 12**). The images have been edited and, currently, we have processed 54%.

The otolith length ranged from 8 to 12.94 mm (mean ± SD = 10.38 ± 1.52 mm) and the otolith weight from 0.0065 to 0.0230 g (0.041 ± 0.0042 g). The otolith weight- length relationship was adjusted to a non-linear model and the parameters were estimated by nonlinear least squares and the slope b was assessing using the Student's t-test to identify the iso or alometric condition. All analyses were performed using the R software. The nonlinear equation for the otolith weight-length relationship is $O_W=0.000499 \times O_L^{1.413}$ (where: O_W – otolith weight; O_L – otolith length), which presented negative allometric growth ($b \neq 3$). The curve representing the length-weight relationship is presented in the **Figure 13**.

The otolith samples were embedded in polyester resin and transverse sections were obtained by a low-speed saw, for the identification of both daily and annual increments (**Figure 14**). For the annual cuts (≈ 350 μm) the sections were attached directly between a slide and a cover slide with polyester resin. For the daily increments the transverse sections (≈ 500 μm), sections were attached to a slide with thermoplastic glue to be grinded with wet sand-paper and polished in plates with water and aluminum

powder until reach the primordium region. There were then shift to the other side to be submitted to the same process. The main goal is to access the viability of both daily and annual readings and for what sizes they will provide acceptable results.

For the Southwest Atlantic, from the 277 sampled otoliths for annual growth analysis, 157 slides were prepared (56%), 35 were already cut (13%), and 87 were embedded to be cut (31%). For the daily growth analysis, we have prepared 5 samples from an expected number of 75 otoliths, which corresponds to 6%. We also prepared 30 slides for the microchemistry analysis, which were sent to the Laboratory for Integrative Fish Ecology, at University of Idaho. A total of 120 otoliths are under processing.

Regarding the samples from another localities, unfortunately, we had suffered from the restrictions due to the Pandemic COVID-19, and samples only arrived in late April 2021, which compromised the work progress. A total of 113 and 99 otoliths from Canary Islands (Northeast Atlantic) and Cote d´Ivoire respectively, arrived in Brazil. Processing of the otoliths from the other regions will only be finalized in the next contract.

2.3. Reproduction parameters

2.3.1 Atlantic Bonito Sarda sarda

Data for BON were obtained from the SMTYP database (2018-2019). A total of 420 fish were used for the preliminary analysis of L_{50} using microscopic staging, and 876 fish were used for the preliminary analysis of L_{50} and spawning season combining macroscopic and microscopic data.

We have analyzed the data grouped by two hypothesis: (1) ICCAT areas: MED (BIL95), NE-ATL (BIL94B), and SE- ATL (BIL 97) and (2) grouped by Stock as suggested by Viñas et al. (2020) - Stock 1: BIL 95+ Portugal (BIL 94B); Stock 2: BIL 94 B, except Portugal and Stock 3: BIL 97 = SE-ATL).

For the analysis, we used the macroscopic and microscopic scales and the protocol for taking macroscopic images agreed in the "Workshop on small tunas biology studies for growth and reproduction" (Saber et al., 2020). For the microscopic analysis, a representative portion of the preserved gonad tissue was dehydrated in ascending concentrations of ethanol, cleared with n-butanol, and embedded in paraffin. Sections were cut at 10 μm and stained with Mallory's trichrome stain. Each individual gonad was histologically classified by two readers. When inconsistencies were detected, a third reader was required. Microscopic maturity stating of gonads of small tuna species was based on a modification of the criteria of Schaefer (1998) and Farley et al. (2013). Length at first maturity were estimated (L_{50}) for each ICCAT area (NE-ATL, SE-ATL, and MED) and Stock (Viñas et al., 2020). We also used combined maturity data (sexes-combined) to estimate the length at first maturity (L_{50}) for each ICCAT area (NE-ATL, SE-ATL, and MED) and Stock (1 and 2). For stock 1 and ICCAT MED area, we calculated the L_{50} for separated sex. When discrepancies in the combined analysis were observed, we correct the macroscopic stage with the microscopic criterion. L_{50} was calculated by fitting the proportion of mature fish to a logistic equation, assuming a binomial error distribution, to model the probability of maturity (p) by length. Confidence intervals for the parameters of the logistic regression were estimated using bootstrapping.

Considering the hypothesis 1 (ICCAT area), only for MED area, it was possible to analyze L_{50} by separated sex. It is necessary more small-sized individuals to fit the logistic curve in the NE-ATL and it was not possible to adjust the logistic curve for the SE-ATL due to the narrow size range of analyzed fish (**Figure 15** and **Table 4**).

Considering the hypothesis of the division of stock proposed by Viñas et al. (2020), as for the ICCAT area, only for stock 1 (MED + Portugal area), it was possible to analyze the L_{50} by sex. For the Stock 2 and 3 was not possible to adjust the logistic due to the absence of small-sized fish histological analyzed (**Figure 16; Table 5)**. **Table 6** shows the L_{50} estimates and confidence levels (95%) for male, female, and sex-combined BON for both MED (ICCAT area) and Stock 1(Viñas et al., 2020). All estimates are in the range of L_{50} previously reported in the area.

To fully achieve the objectives of estimating the L_{50} considering both hypothesis, it is necessary: (a) Increase the number of small-sized individuals in the histologic analysis for the NE-ATL (Stock 2) and SE-ATL (Stock 3); (b) Increase the number of fish > 56 cm FL in the histologic analysis for SE-ATL (Stock 3) and (c) Increase the number of males in the histologic analysis mainly for Stocks 2 and 3 (NE-ATL and SE_ATL).

Figure 17 and **Table 7** show the size distributions of BON by maturity stage (mature: in green; immature: in red) by ICCAT area. In the SE-ATL, the size distribution of mature fish overlaps completely with the size distributions of the immature. For this reason, the logistic curve cannot be adjusted, and we cannot estimate the L_{50}. Using this methodology, we obtained the best L_{50} estimates for the ICCAT MED area (**Figure 18**, **Table 8**). However, for the NE-ATL, L_{50} (all) was estimated as 42.39 cm FL (41.49 – 43.18 cm) (**Figure 19**), but very few small- sized individuals were sampled. This fact could bias the L_{50} estimates. For the SE-ATL, giving the narrow size classes available, this estimate was not possible to be obtained with confidence.

Considering the stock hypothesis (Viñas et al., 2020), in the SE-ATL (Stock 3), the size distribution of mature fish overlaps completely with the size distributions of the immature. For this reason, as for the ICCAT area the logistic curve cannot be adjusted, and hence it is not possible to estimate the L_{50}. **Figure 20** shows the size distributions of BON by maturity stage (mature: green/immature: Red) by stock area and **Table 9** the numbers and size of individuals analysed by stock.

L_{50} was obtained for Stock 1 and 2 (**Figure 21** and **Table 10**). However, there is few samples of small-sized fish for stock 2. This circumstance could bias the L_{50} estimates.

In order to improve the combined micro/macro maturity analysis, we suggest to: (a) increase the number of small-sized individuals in the sampling for the NE-ATL (stock 2) and SE- ATL (Stock 3); (b) Increase the sampling of large-sized fish in the sampling for SE-ATL (stock 3) and (c) Increase the number of immature individuals in the sampling for the NE-ATL (stock 2) and SE-ATL (Stock 3).

Considering the ICCAT area MED, active females (Stages III and IV) are found from March to July; spawning females (stage IV) are found from May to July. No samples are available in April. For the males, active males (Stages III and IV) are found from May to July, spawning males (Stage IV) are found in May and July. Note that we have no samples from April and very few samples from June (10) (**Figure 22**).

In the ICCAT area NE- ATL, active females (Stages III and IV) are found from October to August; spawning females (stage IV) are found from October to July. Note that we have no samples from April. For males, active males (Stages III and IV) are found from October to August, spawning males (Stage IV) are found from August to March. Note that we have no samples from April and very few samples from may (14) and July (10) (**Figure 23**).

In the ICCAT area SE- ATL (also Stock 3 by Viñas et al. 2020), active females (Stages III and IV) are found from May to October; spawning females (stage IV) are observed from May to October, but with an important decline in October. Active males (Stages III and IV) are found from May to October and spawning males (Stage IV), from June to September (**Figure 24**).

Considering the classification proposed by Viñas et al. (2020), for Stock 1, active females (Stages III and IV) are found from March to July); spawning females (stage IV) from May to July. Note that we have no samples from April. Active males (Stages III and IV) are found from May to August and spawning males (Stage IV) in May and July (**Figure 25**). Note that we have no samples from April and very few samples from June (14).

For Stock 2, reproductive Active BON (Stages III and IV) are found from October to August) and spawning individuals (stage IV) from October to August. Note that we have no samples from April (**Figure 26**).

2.3.2 Little tunny Euthynnus alletteratus

Concerning the LTA, it has been completed the analysis and readings of more than 250 LTA for all ICCAT areas. However, given the delay of the arrival of the samples, gonads analysis and reading will be only completed by the next contract and hence the parameters determination.

2.4. Objective C. Refine the stock structure analysis

Once the 2019 SMTYP project with ICCAT finished, the population structure of BON and LTA were mainly determined. However, two questions about the stock structure of these two species were still open. The first question related to the genetic relationships of the locations of Morocco and Mauritania (AT-NE) in the Atlantic bonito distribution. We have concluded that BON presents a complex population structure with the samples of the Mediterranean (MED) locations of Tunis and Spain and that of the AT-NE off Portugal clearly genetically differentiated from the samples of Senegal (AT-NE) and Côte d'Ivoire (AT-SE). The two locations of Morocco and Mauritania (AT-NE) are situated in intermediate genetic situation between these two groups of locations. The second question concerns the LTA genetic population structure. We have found two clearly differentiated genetic pools. All individuals from Senegal, Côte d'Ivoire and Gabon, are grouped together, and separated from the locations of Portugal, Tunis and Spain. The amount of genetic differentiation between these two groups is at species levels. However, the inability to collect samples for this species from the intermediate location, such as Morocco or Mauritania, hinders the identification of the precise geographic boundary of these genetic pools.

Based on these results of the previous years, two objectives were proposed for the current contract (2020-2021), which were partially achieved, mainly due to the impact of the COVID-19 which enormously difficulted to acquisition of the required samples on time to be genetically analyzed.

Objective 1. For BON, determinate the population structure by using genetic markers with large resolution capability with special emphasis in the intermediate location of Morocco and Mauritania;

About 100 individuals of BON representative of the past sampling locations were sent to be genetically analyzed by ddRadSeq. Note, that the individuals from the Morrocco and Mauritania are initially were not included in this analysis since these samples were not available at LIG laboratory of the Universitat de Girona at the moment necessary to send samples for ddRAd sequencing. We are still waiting to have the sequencing results since we had difficulties in having DNA of enough quality together with some delay from the sequencing service. Currently all the quality controls before the ddRAdseq sequencing have been achieved and we are expecting to have the result by end of June or beginning of July.

On May 2021 finally arrived at the LIG laboratory of the Universitat de Girona 20 BON from the intermediate location of Morocco/Mauritania. These samples were analyzed using traditional methods (i.e., genetic variability on the mtDNA Control region; see detailed methodology in the Wahoo section). The genetic variability of these sample is the range of the genetic variability of the bonito samples analyzed up to date (see **Table 11**). No genetic differentiation was detected when the location of Morocco (data 2018 and 2021) were added (Φ_{ST} = 0.146; P-value = 0.000). These results suggest a genetic temporal stability for this area. Similar results of temporal stability were observed is also observed in Bonito for the locations that different year were compared (see document SCRS/2020/038).

Objective 2. For LTA, analyze the intermediated locations of Morocco and Mauritania by using the traditional marker of the mitochondrial control region, to establish the boundary of the two differentiated genetic pools observed in this species.

No new analysis has been done as no new samples from intermediate locations have been provided to the LIG laboratory of the Universitat de Girona. Nevertheless, to genetically confirm the deep genetic differentiation between the LTA locations, a new analysis including additional nuclear genetic markers has been realized. This new analysis included representative samples of the two deep genetically differentiated stocks. The new analysis confirmed the presence of the differentiation at species levels. However, to fully confirm the putative presence of two species, non-genetic methodologies such as growth, maturity and morphological analysis should be added.

Objetive 3 – For WAH, additional analysis of the stock structure of Wahoo, adding the SW-Atlantic. Sub-samples of approximately 2g of 53 individuals were processed by Laboratory of Biology and Fisheries Technology at the Federal Rural University of the Semi-Arid (LABTOP / UFERSA). These

samples were then stored in plastic tubes, fixed in 70% alcohol, and then sent to the University of Girona, in Spain, responsible for the studies of genetics.

During the period of this contract, the Brazilian partner provided 63 additional samples of WAH from Brazil (ICCAT area AT-SW/BIL96) (more than the required 50 in the present Contract). Previous analysis showed lack of genetic heterogeneity of 3 locations belonging to two ICCAT's area AT-SE/BIL97 and AT-NE/BIL94B (SCRS_2020_031).

The genetics methods used were the same that were already described in the previous contract. Using the same methodology facilitate merging all data sets regardless the timing of analysis. 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 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.

Analysis of the sequence variation revealed that four individuals were identified as *Scomberomorus cavalla*. These four individuals were removed for genetic population analysis. The sequence comparison of the 434 bp of the mtDNA-CR of the remaining 272 individuals revealed 186 variable sites. This variability resulted in 212 distinct haplotypes from the 272 sequences (**Table 12**). Accordingly, in all locations the haplotypic diversity was close to one, ranging from 0.993 to 1.000. Nucleotide diversity was also high and similar to the one observed in Atlantic bonito (Viñas et al., 2004; Viñas et al., 2010). This high sequence variation diversity is probably consequence of the presence of two highly divergent groups of sequences (haplogroups) (see **Figure 27**). These two haplogroups were homogenously distributed among localities, ranging from a distribution of haplogroup 1 from 50% in Gabon to 67%. Therefore, this analysis failed to show genetic differentiation among locations with the overall Φ_{ST}= -0.009 (*P-value* = 0.967). Accordingly, no differences were detected in the pairwise comparison among locations (**Table 13**).

The population genetic analysis of Wahoo presents a scenario of homogeneous distribution of genetic variation, which is expected in a species with high migratory potential and large effective population size. To confirm this putative lack of genetic heterogeneity, it should be validated with genetic that present higher power of resolution (ie, genomic makers such as SNPs, RadSeq) than the mtDNA- CR.

3.Problems related to the Contract

Although the team developing this contract was really committed with the project, some important problems made difficult to fully accomplish the objectives. Firstly, and most importantly, there were

serious problems derived from the Covid-19 pandemic. Reduction of the fisheries activities (or even during some months and countries, activity completely ceased), closed laboratories (impeding the data processing and analysis) and shipping delays were determinants to the setback of the sampling arrivals for the area coordinators and hence, samples processing and data analysis. In all three research areas (growth, reproduction and genetics), even considering the great effort carried out by the coordinators, final parameters will be only provided in the next contract. Only a small budget will be required to accomplish these goals.

Moreover, for some species and areas, obtaining the extreme size class are not an easy task and for some cases we could not reach our target. However, this was also partially due to the fisheries restriction given the COVID 19 and we will try to overcome in the next Contract.

4.Final remarks and outcomes

In 2020, the main gaps of sampling for BON and LTA were partially covered and gaps were identified for the next contract. Preliminary results related to the growth and maturity parameters for BON were provided for all areas and for LTA, expect for reproduction, which will be finalized in 2021/2022. Genetic studies were refined as required for this year contract. Additional sample for Southwest WAH were also provided, since this area was barely covered in the previous years. Finally, since very specific gaps were identified for the BON and LTA, the Small tuna Species Group have decided to fill these gaps and finalize the growth and reproduction studies, conclude the analysis for WAH and, given the socio-economic importance, to prioritize other new species for the new cycle of the program: the frigate (FRI) *Auxis thazard* and the bullet tuna (BLT) *Auxis rochei*.

The Small tuna Species Group agreed that the next ToR should focus on a) conduct additional sampling aiming to fill the specific gaps of the biological samples for estimating the growth and maturity parameters of BON, LTA and WAH; b) collect samples for FRI and BLT in the Atlantic and the Mediterranean Sea for stock structure studies, and partially for future studies on age and growth, and reproduction; c) refine the stock structure analysis for WAH and determinate the stock structure analysis for FRI and BLT and d) investigate genetic species differentiation between FRI and BLT.

It is the objective of this Consortium to keep working in a coordinated and cooperative manner to fulfil the objectives established by the SCRS to the SMTYP.

Finally, a note on the demise of the coordinator of the Consortium, Prof. Dr Fábio Hazin, which was another victim of COVID-19. May he rest in peace. His premature departure is a significant loss for the whole team involved in this study. However, the Consortium has nominated as new coordinator Dr Flávia Lucena Fredou, a very close collaborator to Dr Hazin and former rapporteur of the ICCAT SCRS Small Tunas Species Group and coordinator of the ICCAT SMTYP.

Acknowledgements

This work was carried out under the provision of the ICCAT Science Envelope and the ICCAT – European Union Grant Agreement No. SI2.819116 - Strengthening the scientific basis for decision-making in ICCAT, and the ICCAT-US Data Fund.

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. Molecular Ecology Resources. 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.

Farley J.H., Williams A.J., Hoyle S.D., Davies C.R., Nicol S.J., 2013. Reproductive dynamics and potential annual fecundity of South Pacific albacore tuna (Thunnus alalunga). PloS ONE 8 (4), e60577. doi: 10.1371/jounal.pone.0060577

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.

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

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

Ortiz de Zárate, V., Valeiras, J., Ruiz, M., 2007. Sampling protocol for skeletal structures of north Atlantic albacore tuna (Thunnus alalunga) and ageing interpretation. ICCAT Col. Vol. Sci. Pap. 60, 492–506.

Saber, S., Muñoz-Lechuga, R., Macias, D., Ortiz de Urbina, J., Lino, P.G., Diaha, N'G C., Pascual, P., Ngom Sow, F., Angueko, D., Hajjej, G., Baibbat, S'A., Benounnas, K., Medina, A., Quelle, P., Silva, G., Viñas, J., Lucena Frédou, F. 2020. Report on the 2020 ICCAT workshop on small tunas biology studies for growth and reproduction. Collect. Vol. Sci. Pap. ICCAT, 77(9): 100-111

Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution. 4(4):406-425.

Schaefer K.M., 1998. Reproductive biology of yellowfin tuna (Thunnus albacares) in the eastern Pacific Ocean. Inter-American Tropical Tuna Commission Bulletin 21, 205–272.

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. Molecular Phylogenetics and Evolution. 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. Journal of Biogeography. 37(3):557-570.

Table 1. Detailed information on sampling targets by species, size classes and regions. * for WAH no budget was allocated and data collection was opportunistic. LTA - (*Euthynnus alletteratus*), BON (*Sarda sarda*) and WAH (*Acanthocybium solandri*).

Species	Research line	Area	CPCs involved	Target size classes and desirable number of samples (in brackets)
Little Tunny (LTA)	Aging and growth and reproduction	NE Atlantic	Senegal, EU-Spain, EU-Portugal, Morocco	>60 cm (30)
		SE Atlantic	Cote d'Ivoire, Gabon, EU-Spain	≤ 40 cm and > 55 cm (50)
		Med	Tunisia, EU-Spain	≥50 cm (30)
Atlantic Bonito (BON)	Aging and growth and reproduction	NE Atlantic	Senegal, EU-Spain, EU-Portugal, Mauritania, Morocco	≤ 40 cm and > 60 cm (50)
		SE Atlantic	Cote d'Ivoire, Gabon, EU-Spain	≤ 40 cm and > 50 cm (50)
		Med	Tunisia, EU-Spain	< 30 cm and ≥ 50 cm (50)
Wahoo (WAH)	Stock structure	SW Atlantic	Brazil	All sizes (50)*

Table 2 - Number of sampled specimens in the present contract, by species and geographical area. LTA - (*Euthynnus alletteratus*), BON (*Sarda sarda*) and WAH (*Acanthocybium solandri*).

Area	Country	BON	LTA	WAH	Total Geral
ATL-NE	**Mauritania**	12			12
	Morocco	20			20
	Senegal	66			66
	Spain	2	2		4
ATL-NE Total		100	2		102
ATL-SE	**Côte d'Ivoire**		30		30
	Gabon		76		76
ATL-SE Total			106		106
ATL-SW	**Brazil**			90	90
ATL-SW Total				90	90
MED	**Malta**		7		7
	Spain	19	4		23
	Tunisie	26	20		46
MED Total		45	31		76
Total Geral		145	139	90	374

Table 3 - Number of sampled individuals by species, area and "size target" (according to **Table** 1) in the present contract. LTA - (*Euthynnus alletteratus*), BON (*Sarda sarda*) and WAH (*Acanthocybium solandri*).

Species	Area	Country	Other	Large	Small	Undefined	Total
BON	ATL-NE	Mauritania	12	0	0	0	**12**
		Morocco	1	19	0	0	**20**
		Senegal	7	9	50	0	**66**
		Spain	0	0	2	0	**2**
	MED	Spain	0	0	19	0	**19**
	MED	Tunisie	13	10	3	0	**26**
LTA	ATL-NE	Spain	2	0	0	0	**2**
	ATL-SE	Côte d'Ivoire	3	7	20	0	**30**
		Gabon	0	0	76	0	**76**
	MED	Malta	0	7	0	0	**7**
		Spain	0	4	0	0	**4**
		Tunisie	0	20	0	0	**20**
WAH	ATL-SW	Brazil	0	0	0	90	**90**
Total			**38**	**76**	**170**	**90**	**374**

Table 4: Number of Atlantic bonito (*Sarda sarda*) processed by histology by sex and ICCAT area. Size range (FL cm.)

Area	N	N Female	Size Range F	N Male	Size Range M
MED	239	142	25.5 - 71.5	97	26.7 - 53.7
NE-ATL	102	90	37.6 - 67.8	12	39.6 - 53.3
SE-ATL	79	77	37.3 - 56.0	2	43.3 - 43.6

Table 5: Number of Atlantic bonito (*Sarda sarda*) histologically processed by sex and stock (Viñas et al., 2020). Size range (FL cm). Stock 1: BIL 95+ Portugal (BIL 94B); Stock 2: BIL 94 B, except Portugal and Stock 3: BIL 97 = SE-ATL.

Stock	N	N Female	Size Range F	N Male	Size Range M
1	275	167	25.5 - 71.5	108	26.7 - 53.7
2	66	65	43. 0 - 67.8	1	53.3 - 53.3

Table 6 – Length at first maturity for Atlantic bonito (*Sarda sarda*) obtained by microscopic analysis only for MED (ICCAT area) and stock 1 (based on Viñas et al., 2020). (Stock 1: BIL 95+ Portugal (BIL 94B); MED: BIL 95).

Area	Sex	L_{50}	95%LL	95%UL
MED	all	41.37	40.75	42.00
MED	female	41.25	40.45	42.07
MED	male	41.60	40.59	42.46
Stock 1	all	41.63	40.96	42.32
Stock 1	female	41.83	40.94	42.71
Stock 1	male	41.24	40.29	42.13

Table 7: Number of Atlantic bonito (*Sarda sarda*) available for combined (Macro/micro) maturity analysis by sex and area. Size range (FL cm.)

Area	N	N Female	Size Range F	N Male	Size Range M
MEDI	329	211	25.5 - 71.5	118	26.7 - 64.4
NE-ATL	393	215	36.2 - 67.8	178	35.6 - 62.9
SE-ATL	154	85	37.3 - 56.0	69	39.3 - 51

Table 8 - Length at first maturity obtained for Atlantic bonito (*Sarda sarda*) by combined analysis only for MED (ICCAT area B95).

ICCAT Area/stock	Sex	L_{50}	95%LL	95%UL
MED	all	41.31	40.82	41.84
MED	female	41.43	40.81	42.11
MED	male	41.10	40.21	41.92

Table 9: Number of Atlantic bonito (*Sarda sarda*) available for combined (Macro/micro) maturity analysis by sex and stock. Stock 1: BIL 95+ Portugal (BIL 94B); MED: BIL 95. Size range (FL cm.)

Stock	N	N Female	Size Range Female	N Male	Size Range Male
1	441	274	25.5 - 71.5	167	26.7 - 64.4
2	281	152	36.2 - 67.8	129	35.6 - 62.9
3	154	85	37.3 - 56.0	69	39.3 - 51

Table 10 - Length at first maturity obtained by combined analysis for Stock 1 and 2. Stock 1: BIL 95+ Portugal (BIL 94B); Stock 2: BIL 94 B, except Portugal.

Area/Stock	Sex	L_{50}	95%LL	95%UL
Stock 1	all	41.86	41.34	42.37
Stock 2	all	40.76	37.19	42.82

Table 11. Results of Atlantic bonito sampling and molecular diversity indices by year of sampling. In bold the new samples included for the 2020 contract. N, number of individuals; M, number of haplotypes; *h*, haplotypic diversity; π, nucleotide diversity.

Location/	ICCAT area	Code	Year	N	*M*	*h*±*SD*	π±*SD*
Spain	MD/BIL95	ESP2018	2018	108	76	0.985 ± 0.005	0.070 ± 0.034
Spain	MD/BIL95	ESP2019	2019	96	54	0.960 ± 0.013	0.066 ± 0.032
Portugal	AT-NE/BIL94B	PRT2018	2018	65	46	0.975 ± 0.010	0.069 ± 0.034
Portugal	AT-NE/BIL94B	PRT2019	2019	38	28	0.979 ± 0.012	0.067 ± 0.034
Tunisia	MD/BIL95	TUN2018	2018	49	30	0.974 ± 0.010	0.066 ± 0.033
Morocco	AT-NE/BIL94B	MAR2018	2018	40	28	0.968 ± 0.016	0.048 ± 0.024
Mauritania	AT-NE/BIL94B	MRT2019	2018	48	45	0.996 ± 0.005	0.047 ± 0.024
Senegal	AT-NE/BIL94B	SEN2018	2018	49	43	0.990 ± 0.009	0.039 ± 0.020
Cotê d'Ivoire	AT-SE/BIL97	CIV2018	2018	50	38	0.975 ± 0.013	0.017 ± 0.009
Cotê d'Ivoire	AT-SE/BIL97	CIV2019	2019	72	51	0.975 ± 0.010	0.032 ± 0.016
Morocco	**AT-NE/BIL94B**	**MAR2021**	**2021**	**20**	**16**	**0.963 ± 0.033**	**0.053 ± 0.0008**

Table 12. Summary of Wahoo sampling and results of molecular diversity indices. Year, year of sampling. N, number of individuals; M, number of haplotypes; *h*, haplotypic diversity; π, nucleotide diversity

Locality	ICCAT Area	Code	Year	n	M	*h* ± *SD*	π ± *SD*	*% haplgrp 1*
Cotê d'Ivoire	AT-SE/BIL97	CIV	2018-2019	133	114	0.974 ± 0.011	0.077 ± 0.038	65%
Gabon	AT-SE/BIL97	GAB	2018	18	17	0.993 ± 0.021	0.082 ± 0.042	50%
Spain	AT-NE/BIL94B	ESP	2019	62	60	0.999 ± 0.003	0.077 ± 0.038	58%
Brazil	AT-SW/BIL96	BRA	2020	59	56	0.998 ± 0.003	0.079 ± 0.038	60%
All				272	212	0.998 ± 0.001	0.077 ± 0.037	59%

Table 13. Pairwise genetic differentiation among Wahoo samples. Below diagonal, Φ_{ST}s values. Above diagonal, *P*-values. Samples code as **Table 1**.

	CIV	GAB	ESP	BRA
Cotê d'Ivoire	--	0.449	0.995	0.678
Gabon	-0.003	--	0.649	0.642
Spain	-0.006	-0.010	--	0.788
Brazil	-0.003	-0.009	-0.007	--

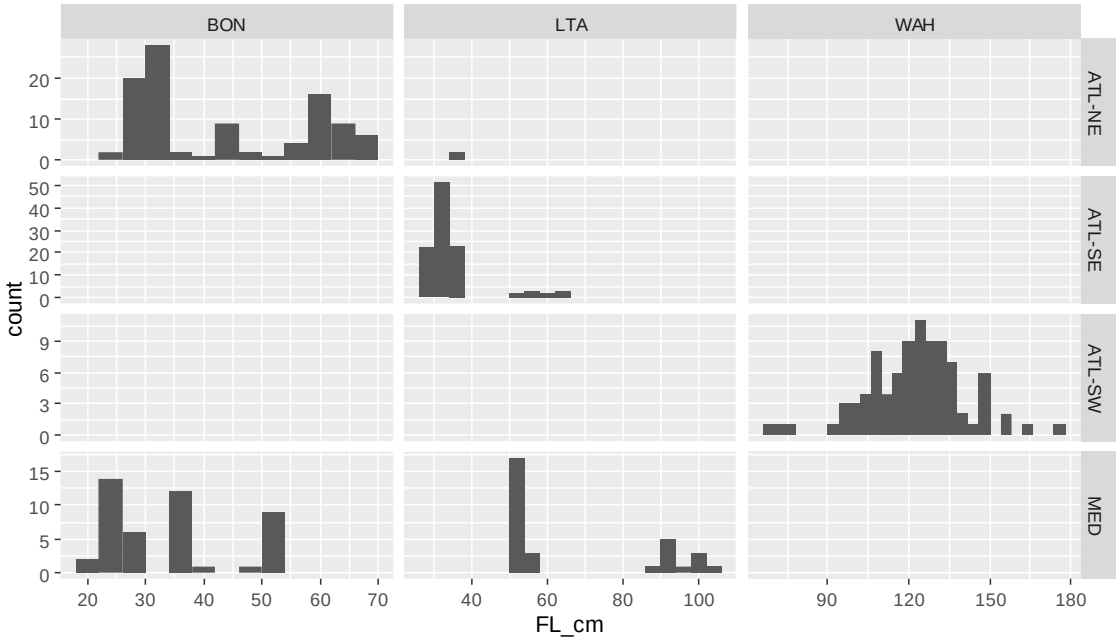

Figure 1- Length distribution of individuals by species and area, sampled during the present contract. LTA - (*Euthynnus alletteratus*), BON (*Sarda sarda*) and WAH (*Acanthocybium solandri*).

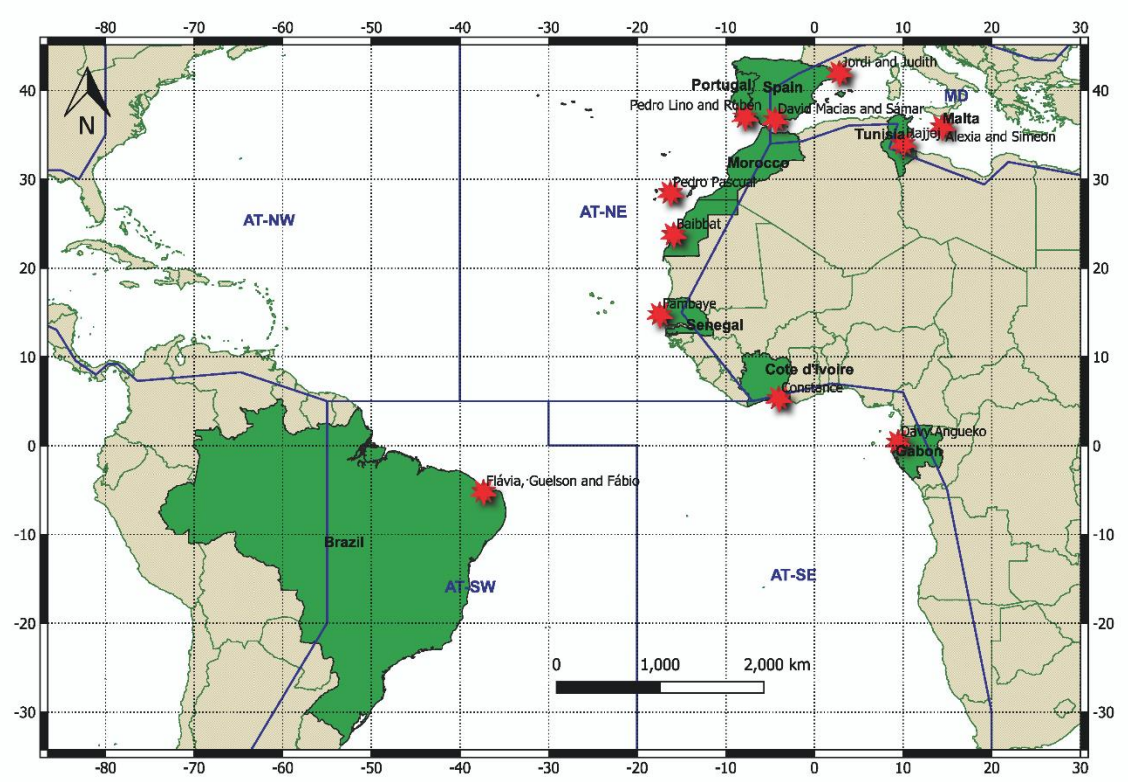

Figure 2. Map with the location of the sampling sites currently available for the age and growth study.

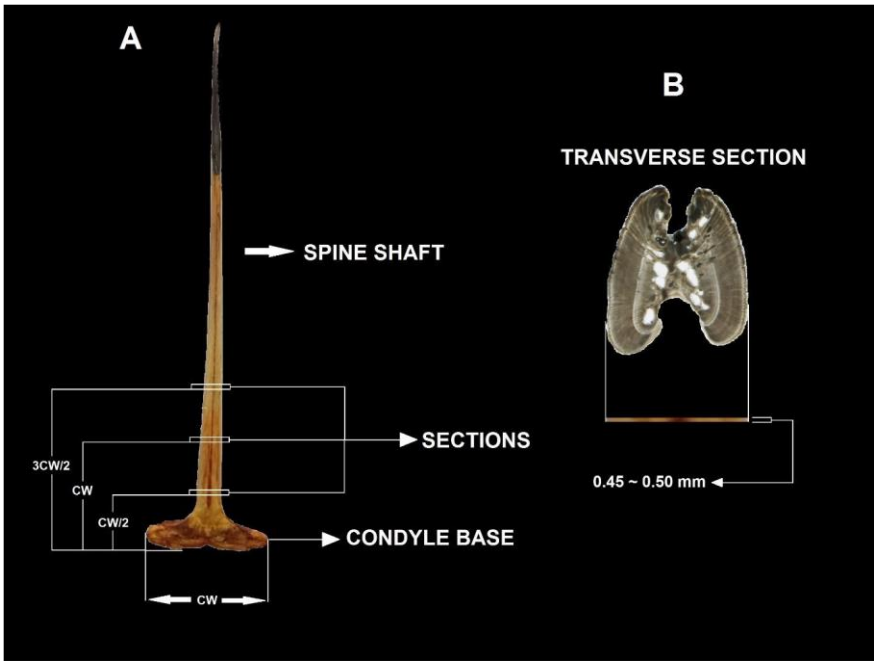

Figure 3. First dorsal fin spine of a small tuna, showing A) the condyle base and location of sections at one and half distance of the width of the condyle base (3CW/2), one distance of the width of the condyle base (CW), and half distance of the width of the condyle base (CW/2) and B) Cross section of the fin spine at distance CW.

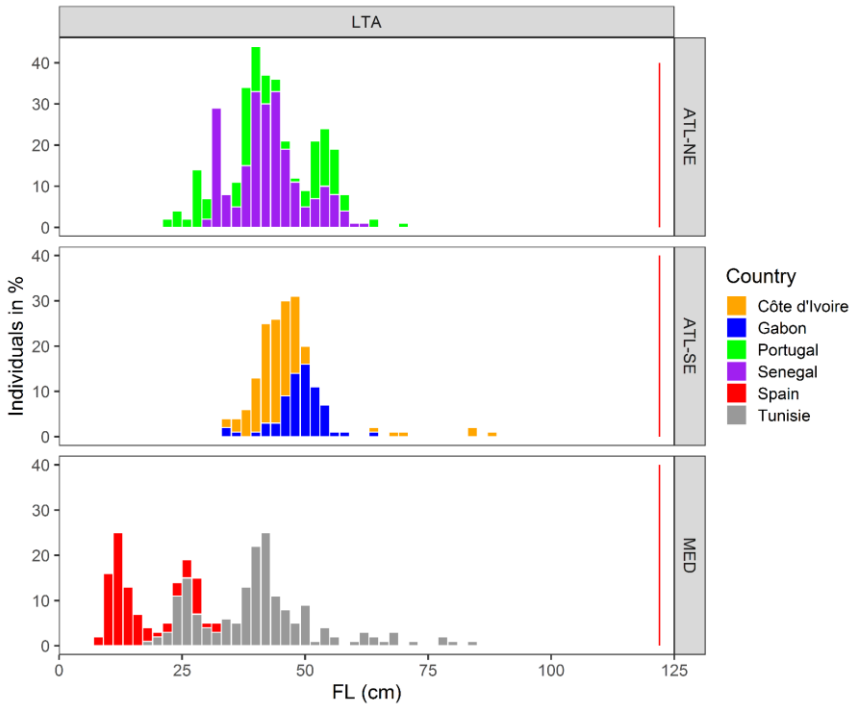

Figure 4. Distribution of the fork length by size classes for little tunny *Euthynnus alletteratus* spine samples currently collected for the age and growth study, for the north-east and south-east Atlantic (separated at the 5ºN) and Mediterranean Sea.

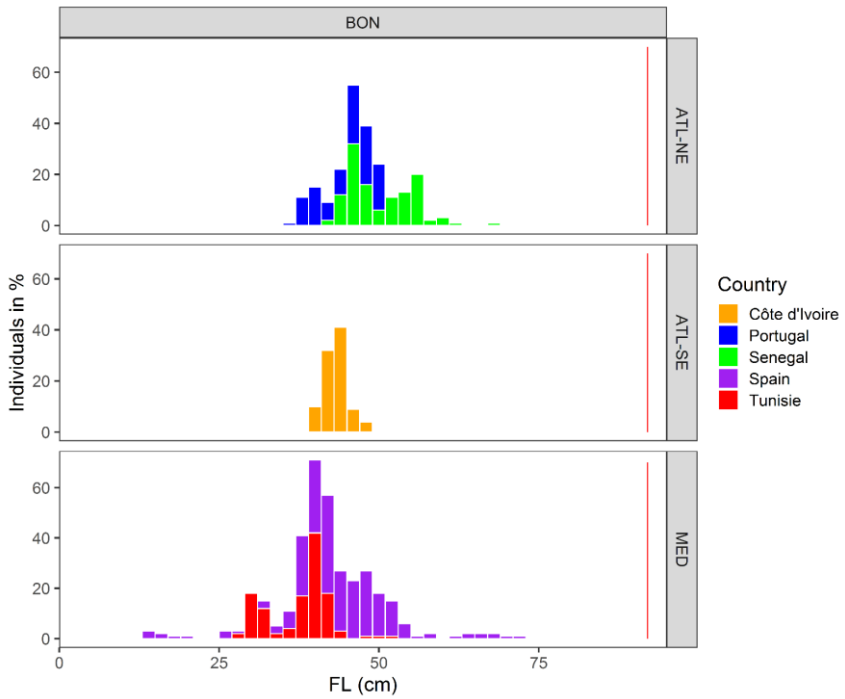

Figure 5. Distribution of the fork length by size classes for of Atlantic bonito *Sarda sarda* spine samples currently collected for the age and growth study, for the north-east and south-east Atlantic (separated at the 5ºN) and Mediterranean Sea.

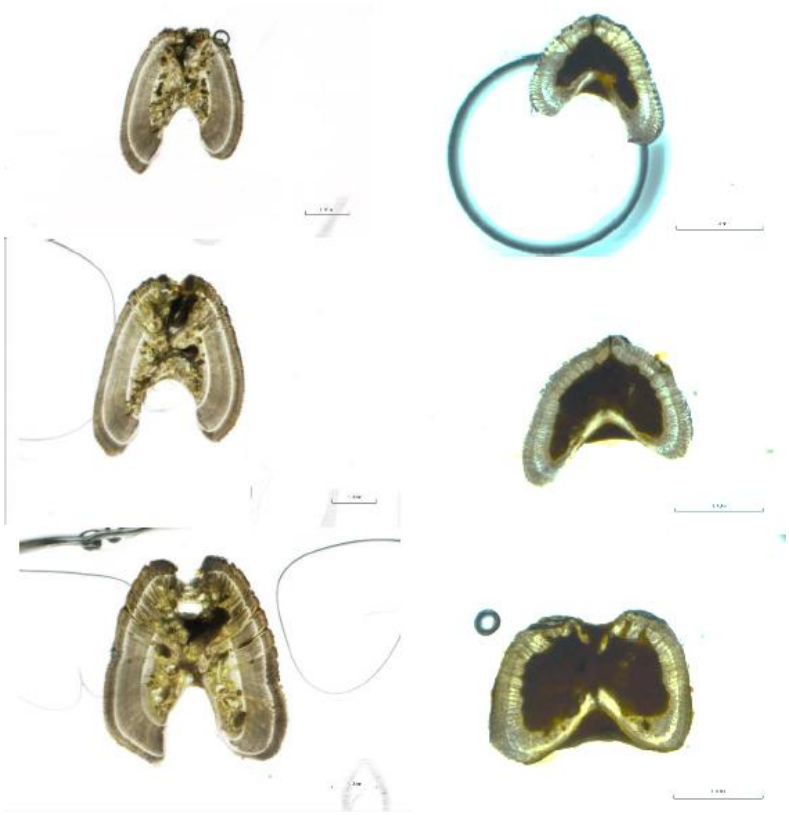

Figure 6. Image analysis sections of two small tuna spines: little tunny *Euthynnus alletteratus* (Left images) and Atlantic bonito *Sarda sarda* (Right images).

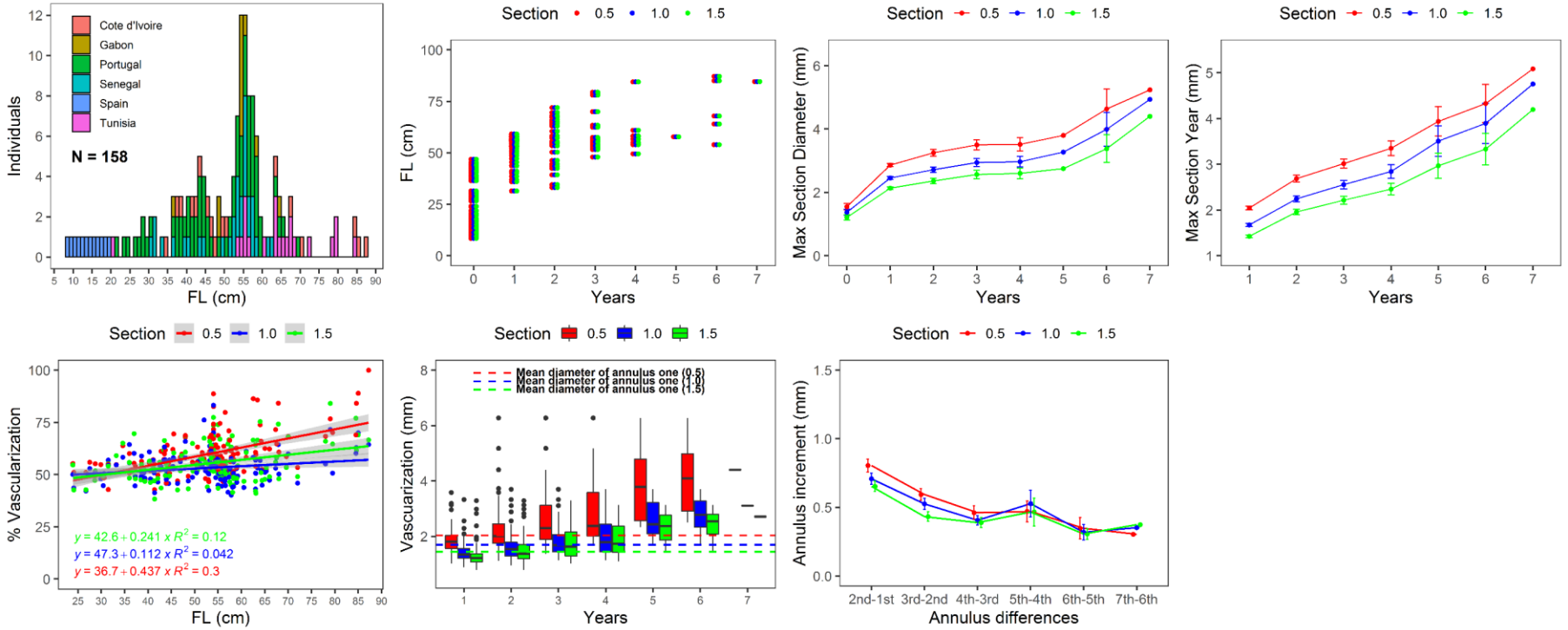

Figure 7. Image analysis of spine sections of little tunny *Euthynnus alletteratus*. Reading of *annulus*, section diameters, vascularization, loss of *annulus* due to vascularization, distance of *annulus* formation and difference in distances between *annulus*.

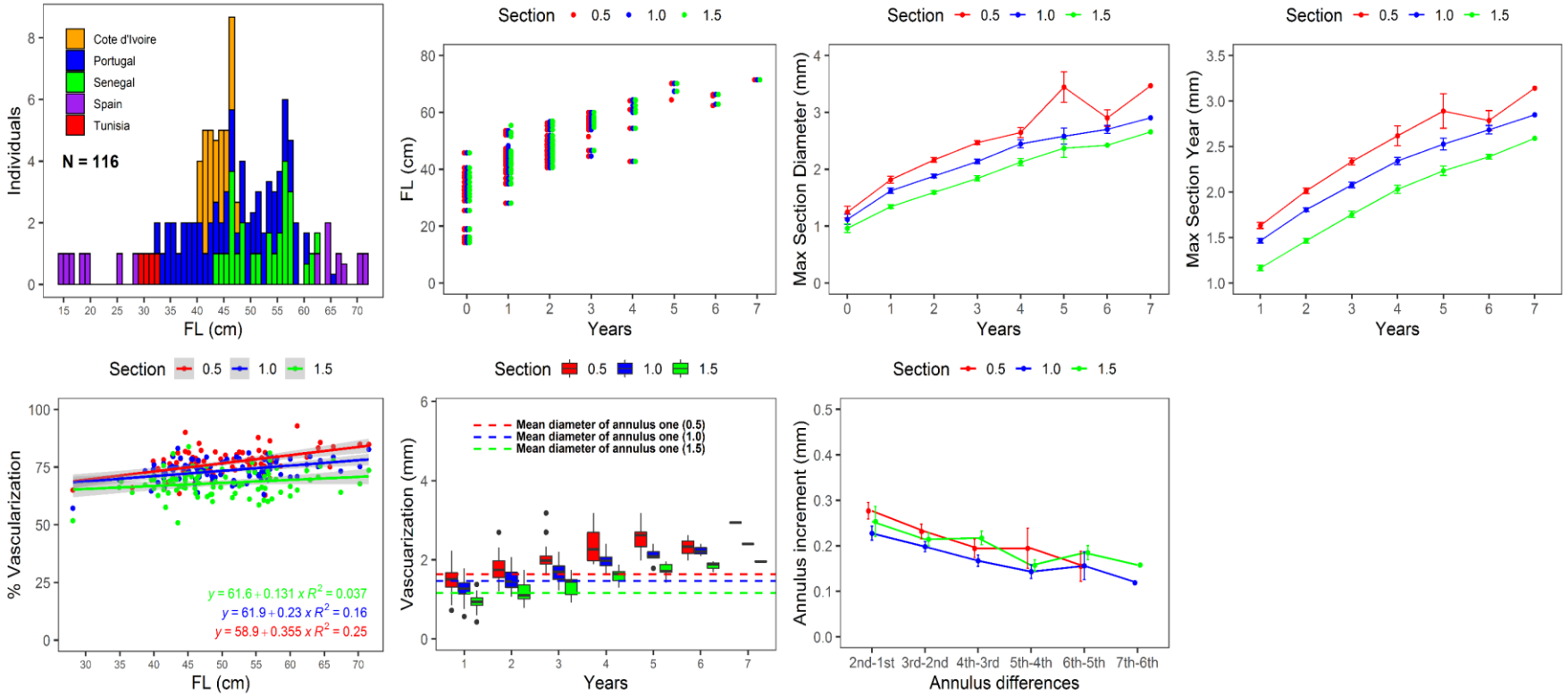

Figure 8. Image analysis of spine sections of Atlantic bonito *Sarda sarda*. Reading of *annulus*, section diameters, vascularization, loss of *annulus* due to vascularization, distance of *annulus* formation and difference in distances between *annulus*.

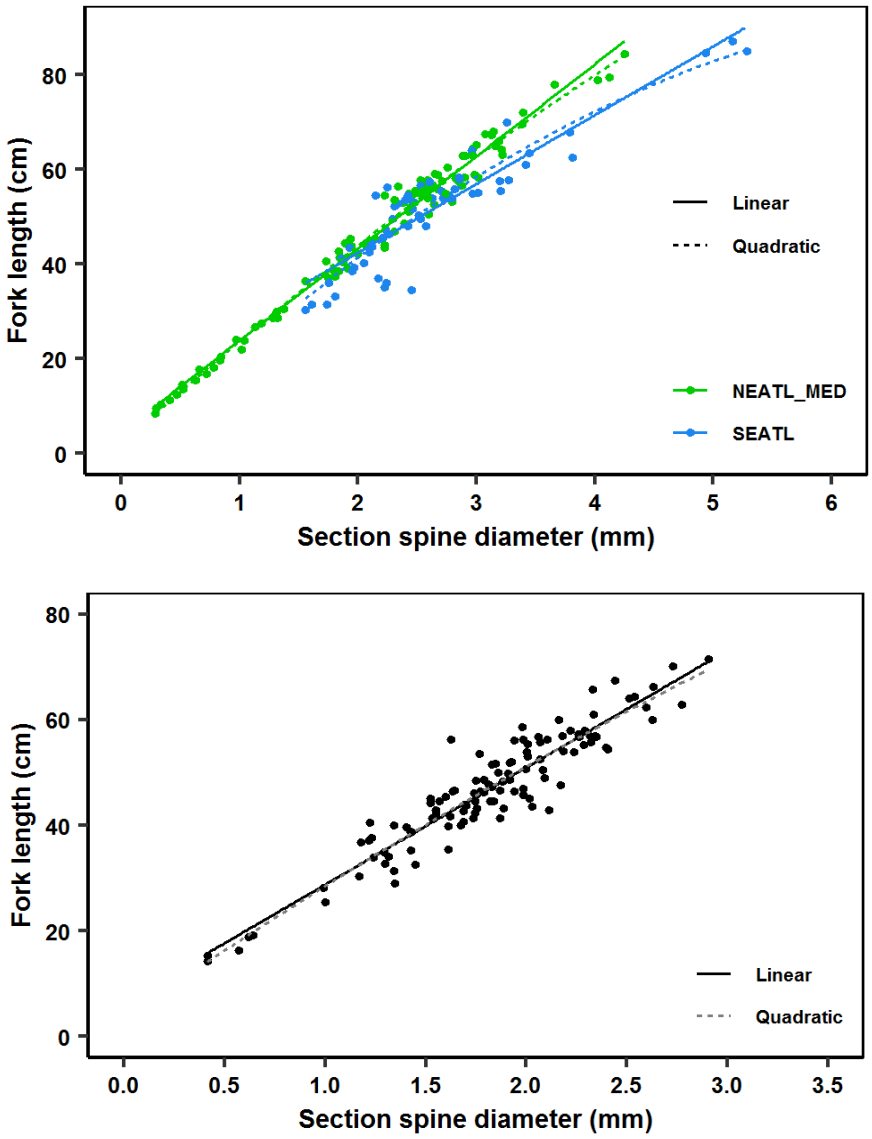

Figure 9. Section spine diameter (mm) relationship to fish size (fork length – FL cm) for little tunny *Euthynnus alletteratus* (Upper panel) and Atlantic bonito *Sarda sarda* (bottom panel).

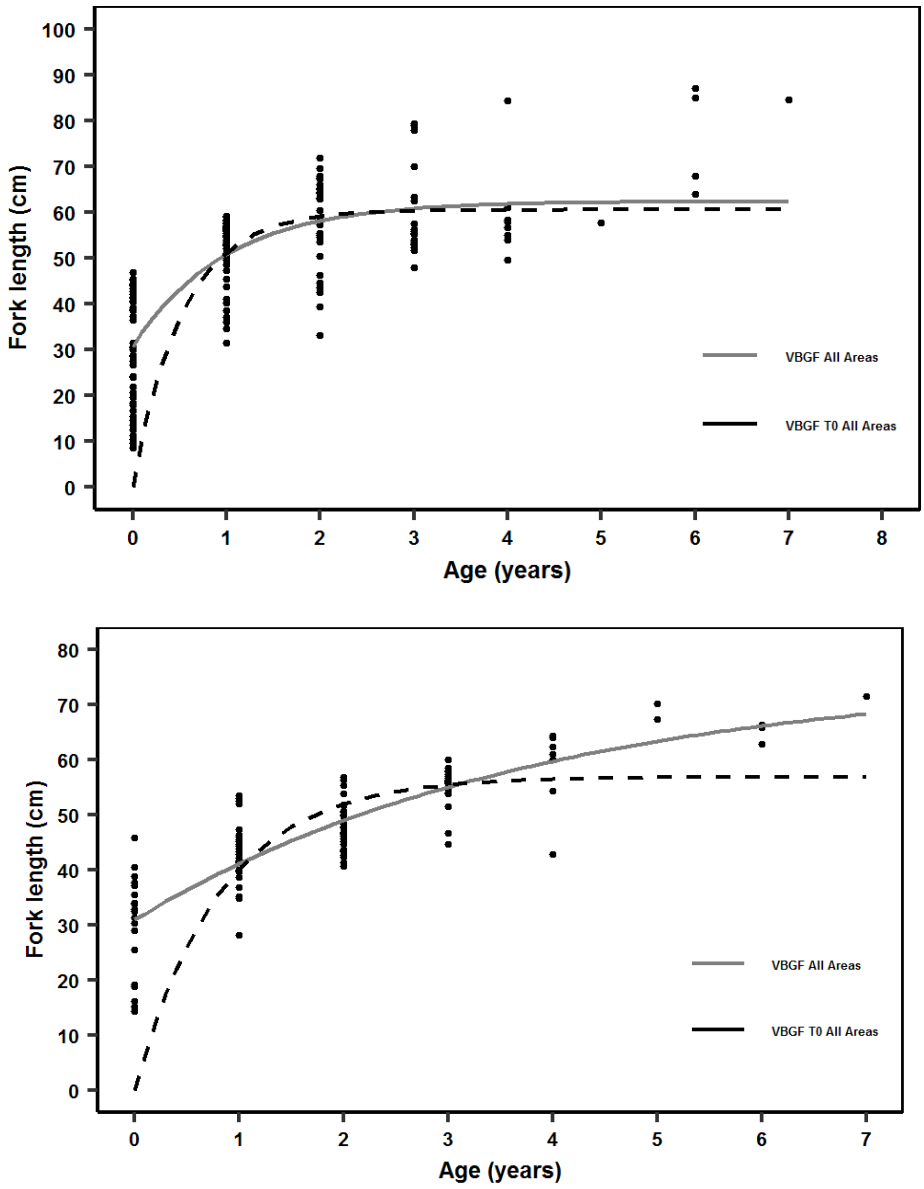

Figure 10. von Bertalanffy growth model applied for both species for little tunny *Euthynnus alletteratus* (Upper panel) and Atlantic bonito *Sarda sarda* (bottom panel).

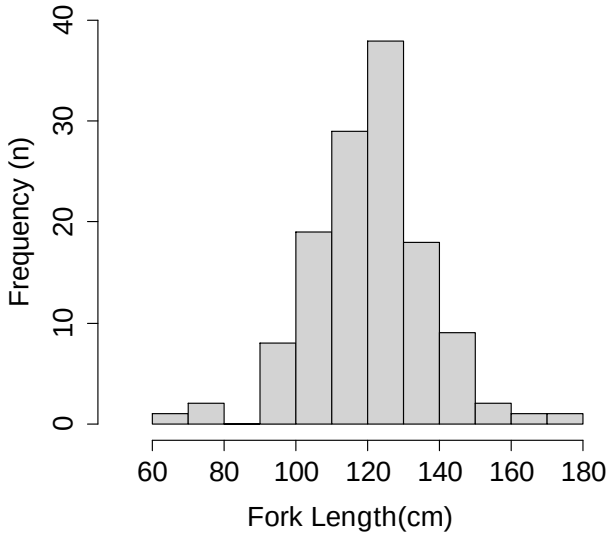

Figure 11. Size distribution of Wahoo (*Acanthocybium solandri*) sampled by the Brazilian team in the Southwestern Atlantic Ocean.

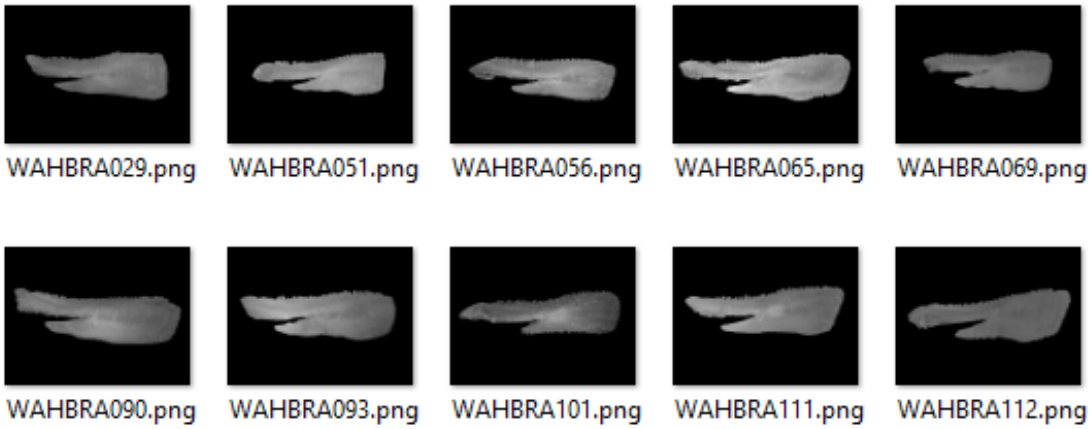

Figure 12. Edited photographs from otoliths of Wahoo (*Acanthocybium solandri*) collected in the southwestern Atlantic Ocean for shape analysis.

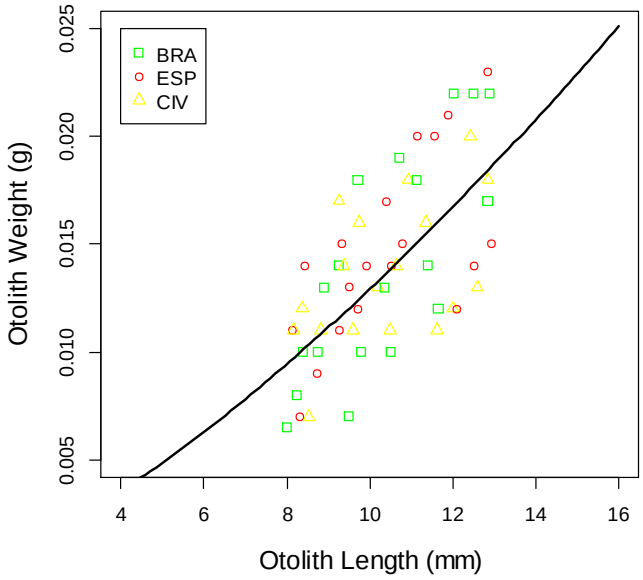

Figure 13. Length-weight relationship from otolith of *Acanthocybium solandri* sampled in the Atlantic Ocean (BRA - Brazil; ESP – Canary Islands/Spain; CIV – Côte d'Ivoire).

Figure 14. Transverse sections from *Acanthocybium solandri* otoliths collected in the southwestern Atlantic Ocean, for the identification of annual (a) and daily (b) increments. P: *primordium*; CP: daily counting path.

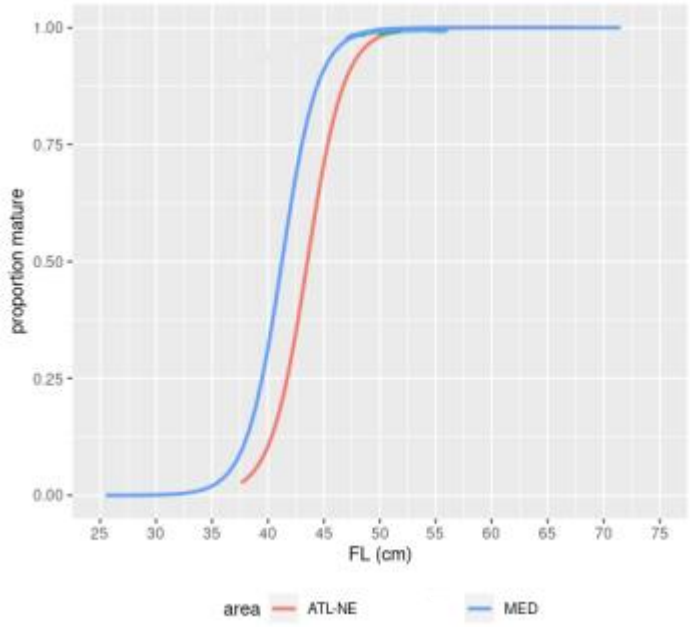

Figure 15 – Logistic curve estimated for Atlantic bonito *Sarda sarda* female maturity considering the ICCAT areas (ATL-NE and MED).

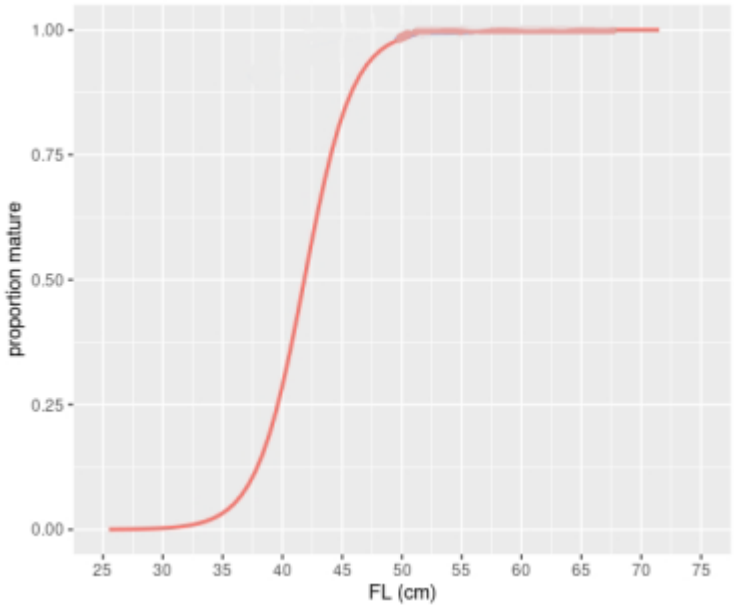

Figure 16 – Logistic curve estimated for female of Atlantic bonito (*Sarda sarda*) considering the Stocks BIL 95+ Portugal (BIL 94B) areas;

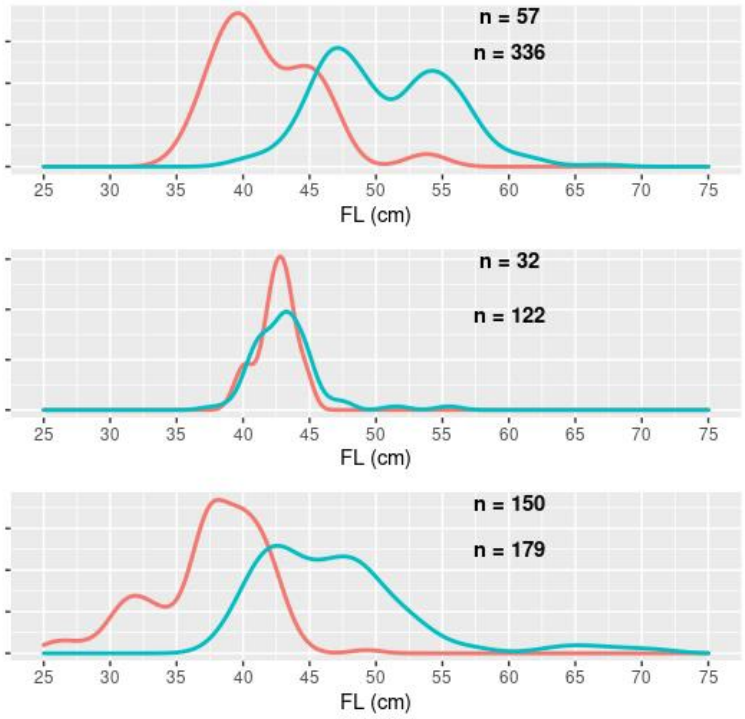

Figure 17. Size distributions of Atlantic bonito (*Sarda sarda*) by maturity stage (mature: green/immature: Red) per area. The upper panel corresponds to the NE-ATL area. The medium panel corresponds to the SE-ATL area. The lower panel corresponds to the MED area. n= sample size of Immature (top value) and Mature (bottom value).

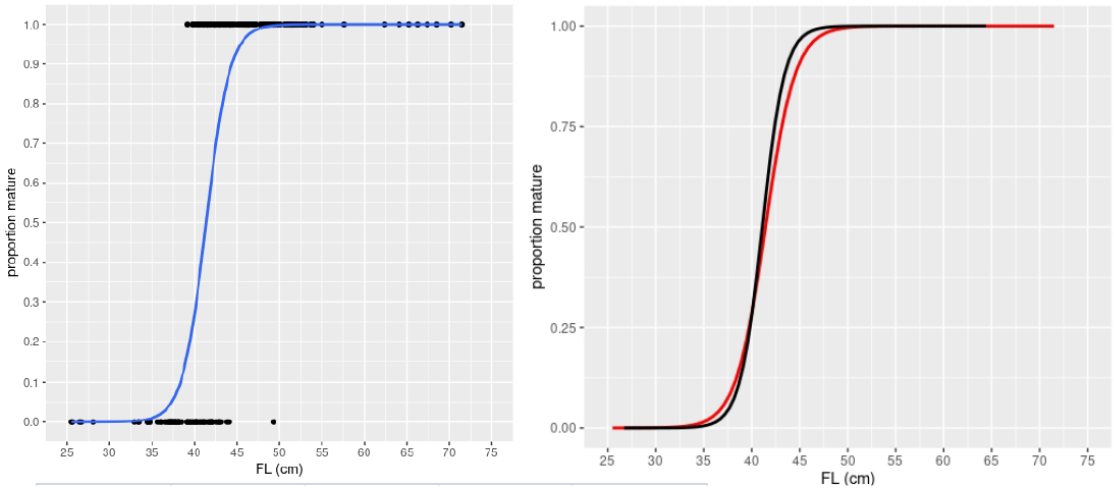

Figure 18 - Logistic curve estimated for Atlantic bonito *Sarda sarda* considering in the MED ICCAT areas. Left panel - estimate for sexes combined, right panel estimate by sex (red female, black male).

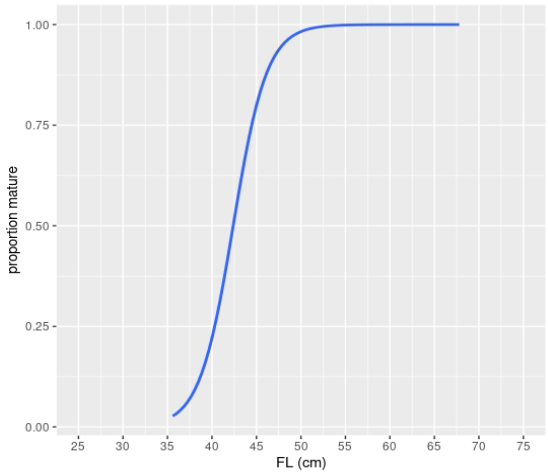

Figure 19 – Logistic curve estimated for Atlantic bonito (*Sarda sarda*) considering in the NE-ATL for sexes combined.

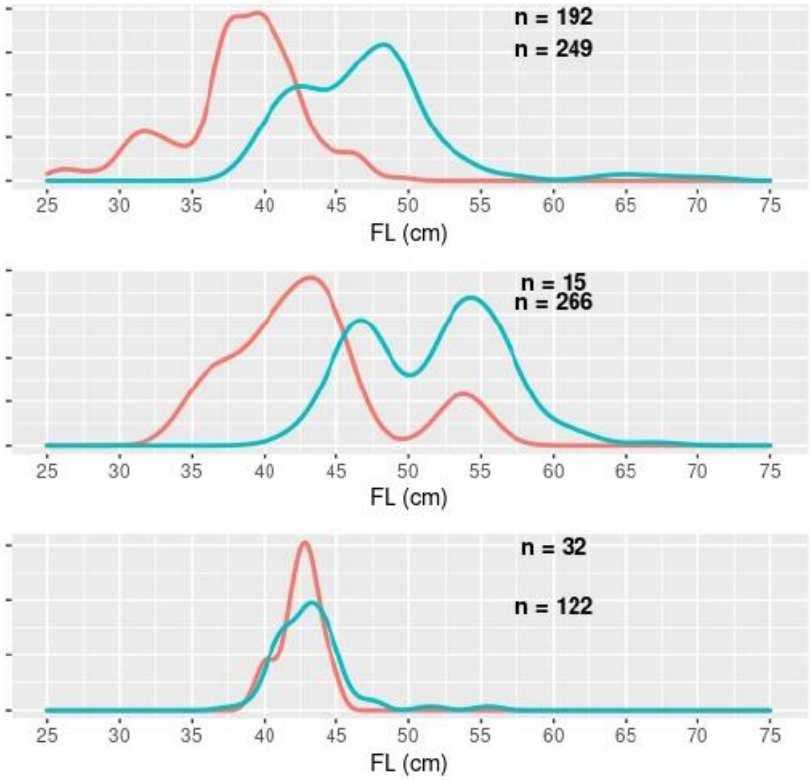

Figure 20 - Size distributions of Atlantic bonito (*Sarda sarda*) by maturity stage (mature: in green and immature: in red) per stock (Viñas et al., 2020). The upper panel corresponds to stock 1. The intermediate panel corresponds to stock 2. The lower panel corresponds to stock 3 (SE-ATL). Stock 1: BIL 95+ Portugal (BIL 94B); MED: BIL 95. n= sample size of Immature (top value) and Mature (bottom value).

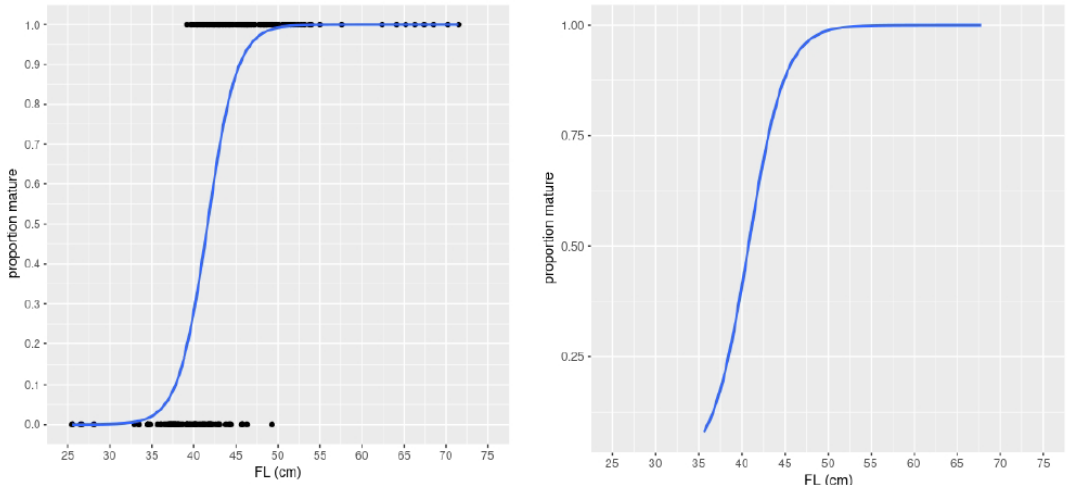

Figure 21 – Logistic curve estimated for Atlantic bonito (*Sarda sarda*) sex-combined for stock 1 (left panel), and stock 2 (right panel). Stock 1: BIL 95+ Portugal (BIL 94B); Stock 2: BIL 94 B, except Portugal.

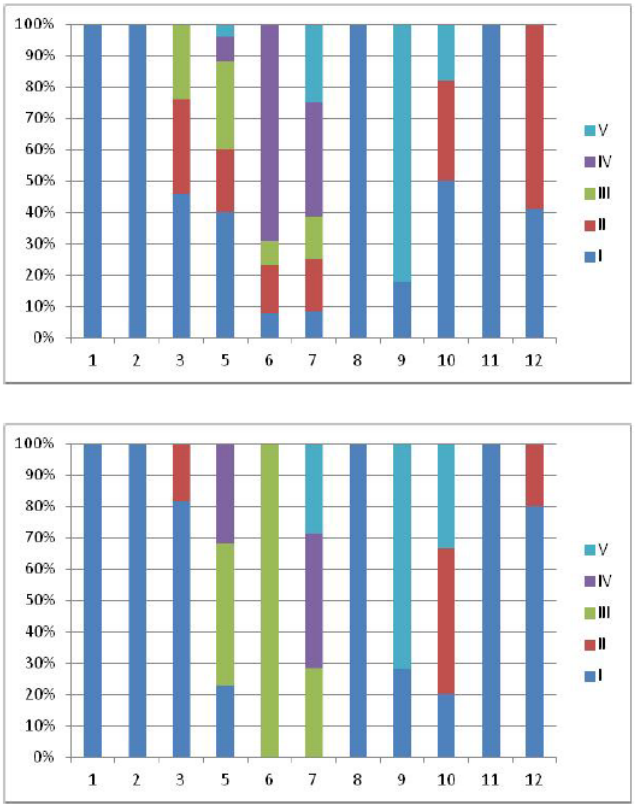

Figure 22 - Maturity stage by month for Atlantic bonito (*Sarda sarda*) in the Mediterranean ICCAT area. Upper panel – female; lower panel – male.

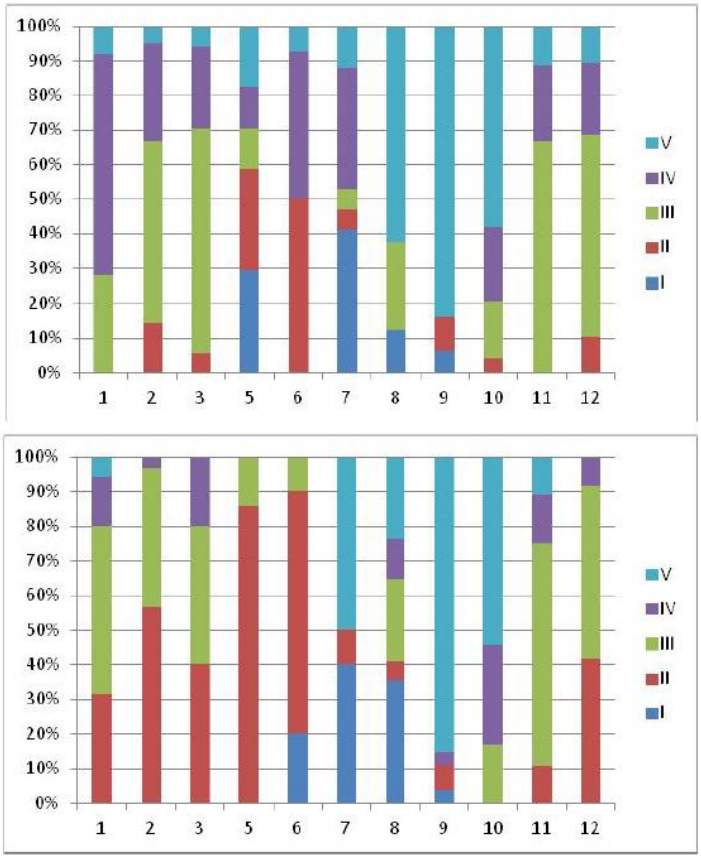

Figure 23 - Maturity stage by month for Atlantic bonito (*Sarda sarda*) of NE-ATL ICCAT area. Upper panel – female; lower panel – male.

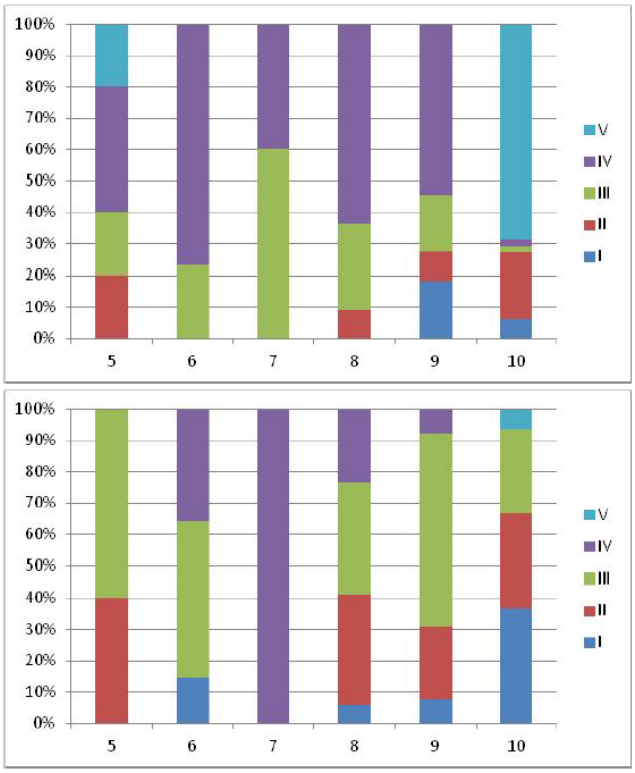

Figure 24 - Maturity stage by month for Atlantic bonito (*Sarda sarda*) of SE-ATL ICCAT area. Upper panel – female; lower panel – male.

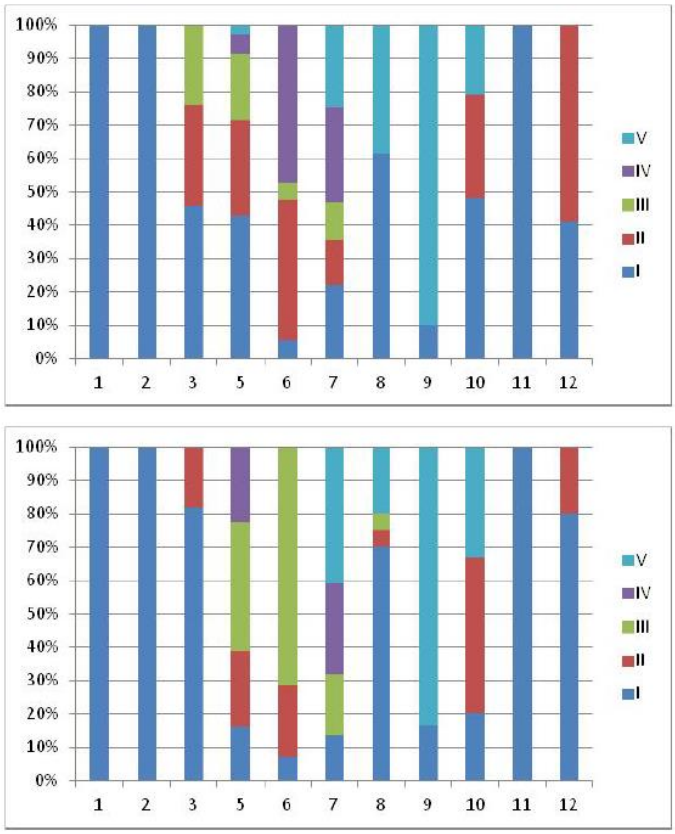

Figure 25 - Maturity stage by month for Atlantic bonito (*Sarda sarda*) for Stock 1 (following Viñas et al. 2020). Upper panel – female; lower panel – male.

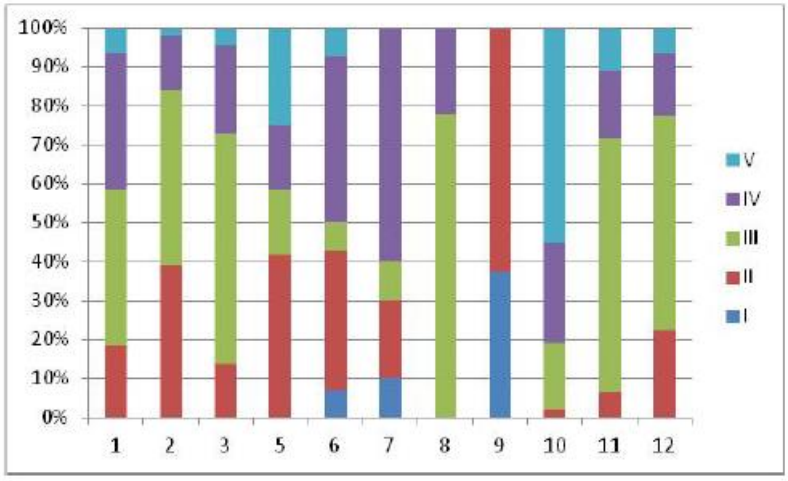

Figure 26 - Maturity stage by month for female of Atlantic bonito (*Sarda sarda*) of Stock 2(following Viñas et al. 2020).

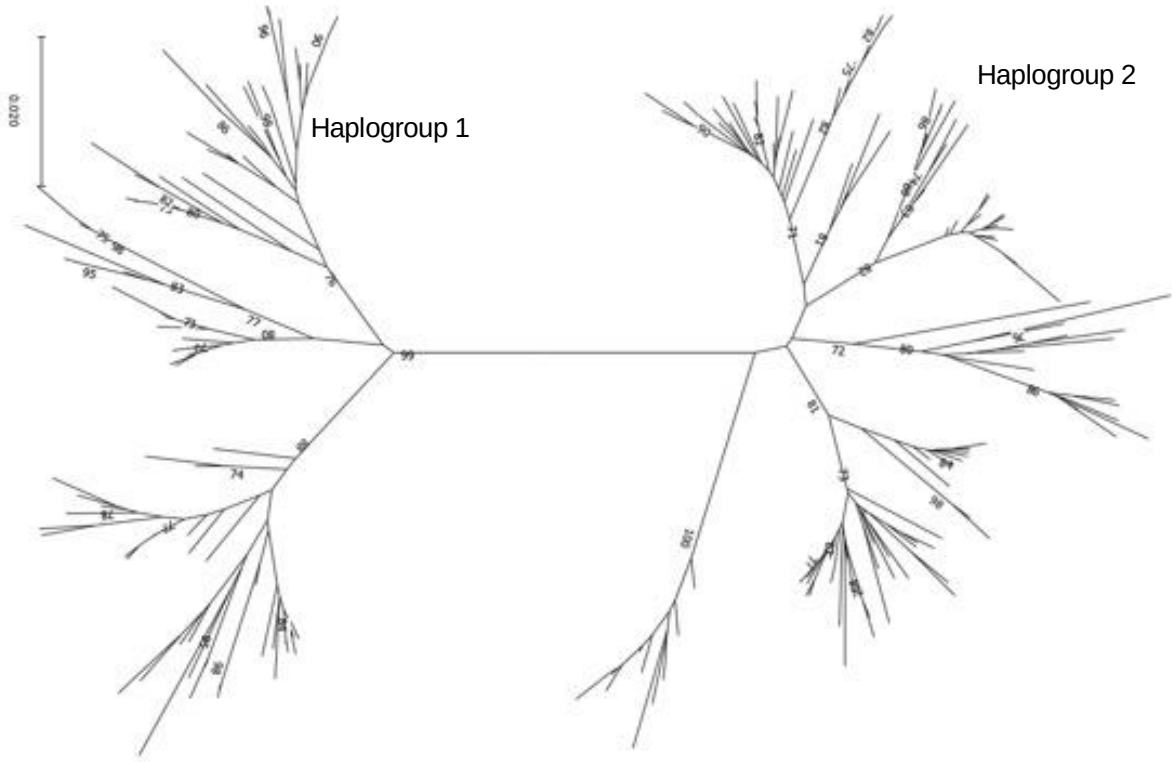

Figure 27. Unrooted phylogenetic tree of the 212 Wahoo mtDNA-CR haplotypes. Values in branches are bootstrap percentages above 70% consistency.