FEASIBILITY STUDY FOR THE APPLICATION OF THE CLOSE KIN MARK RECAPTURE METHODOLOGY FOR EASTERN ATLANTIC AND MEDITERRANEAN BLUEFIN TUNA.

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SUMMARY

This paper examines the feasibility of applying the close-kin mark-recapture (CKMR) methodology to eastern Atlantic and Mediterranean bluefin tuna (BFT-E). The vision and purpose are briefly described and the state of the art of the different elements necessary to apply this methodology is presented: modeling and evaluation of alternative sampling designs, sampling characteristics and possibilities, genetic techniques such as genotyping and epigenetic aging, identification and modeling of kinship data, management and state of the art of the possible project, budget estimation and future integration with the existing CKMR for western BFT.

RÉSUMÉ

Ce document examine la faisabilité de l'application de la méthodologie de marquage-recapture de spécimens étroitement apparentés (CKMR) au thon rouge de l'Atlantique Est et de la Méditerranée (BFT-E). La vision et l'objectif sont brièvement décrits et l'état actuel art des différents éléments nécessaires à l'application de cette méthodologie est présenté : modélisation et évaluation des plans d'échantillonnage alternatifs, caractéristiques et possibilités d'échantillonnage, techniques génétiques telles que le génotypage et la détermination de l'âge épigénétique, identification et modélisation des données de parenté, gestion et état des lieux du projet éventuel, estimation du budget et intégration future avec la CKMR existante pour le thon rouge de l'Ouest.

RESUMEN

Este documento examina la viabilidad de aplicar la metodología de marcado y recaptura de individuos estrechamente emparentados (CKMR) al atún rojo del Atlántico oriental y Mediterráneo (BFT-E). Se describen brevemente la visión y el propósito y se presentan los datos más recientes sobre los distintos elementos necesarios para aplicar esta metodología: modelización y evaluación de diseños de muestreo alternativos, características y posibilidades de muestreo, técnicas genéticas como genotipado y determinación de la edad epigenética, identificación y modelización de datos de parentesco, gestión e información de última hora sobre el posible proyecto, estimación presupuestaria y futura integración con el modelo CKMR existente para el atún rojo occidental.

KEYWORDS

Close-kin mark-recapture; genetics; kinship; abundance estimation; Atlantic bluefin tuna

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1. Introduction

The method of close-kin mark-recapture (CKMR) has been described as an emerging tool to estimate population parameters (Casas & Saborido-Rey 2023). The CKMR method is based on the premise that the genetic information of an individual can be considered a "mark" that may be "recaptured" in the population of its potential parents by identifying parent-offspring pairs (POPs). Such recaptures are less likely to happen when the population is bigger, so the number and pattern of POPs provide information on adult abundance in a mark-recapture modeling. Likewise, the number of half-sibling pairs (HSPs) that may be encountered also provides information about the size of the parental population and other demographic parameters (Bravington *et al*. 2016b). This method has been successfully applied for almost 10 years in a species of the genus Thunnus, southern bluefin tuna, and results are beginning to be obtained for western Atlantic bluefin tuna. (Bravington *et al*. 2016a; SCRS/P/2024/110).

The objective of this paper is to examine the feasibility of applying the CKMR methodology to bluefin tuna from the East Atlantic and Mediterranean Sea (E-BFT). The vision and purpose are briefly described and the state of the art of the different elements necessary to apply this methodology is presented: The modeling and evaluation of alternative sampling designs, the genetic techniques such as genotyping and epigenetic age, the identification and modeling of kinship data, the management, cost planning of a project of such magnitude and future integration with existing CKMR for W-BFT.

2. Vision/Purpose. One page doc

Eastern Atlantic Bluefin Tuna Close-Kin Mark Recapture Implementation Plan Proposal

Vision: To reduce the greatest source of uncertainty in the Atlantic bluefin management strategy evaluation (MSE) through use of Close-Kin Mark Recapture (CKMR) to inform the absolute scale of the spawning stock biomass, starting with the MSE reconditioning in 2027.

Purpose and need: The recent adoption of a management procedure (MP) provides management stability; however, there is still a key source of uncertainty in the scale of the total population size. Now is the time to embark upon transformative science to apply CKMR methodology to estimate the total spawning stock biomass in time to inform the 2027 reconditioning of the MSE and to support long-term management of Atlantic bluefin, regardless of stock of origin.

Objective: The project has two phases: a 3-year pilot project intended to provide an initial estimate of total BFT-E spawner population size to inform the 2027 MSE review and a longer-term operational project intended to provide a more precise estimate with options for development of CKMR-based management procedures (MPs) for both East and West by 2030. This proposal seeks to obtain support for the 3-year pilot project, which will provide a prerequisite foundation for a longer-term plan.

Why CKMR: The Operating Models considers a wide range of abundance between 200-400kT, which means that catch recommendations may be precautionary for low-biomass scenarios, possibly missing opportunities for higher catches. Accurate biomass estimations allow a more effective management procedure, ensuring that TACs can be closer to the highest sustainable level while safely avoiding overfishing. Unlike most conventional fisheries-derived data, CKMR is not susceptible to changes in fish spatial distribution and fleet behaviour, making it well suited to coping with impacts of climate change. In the long-term, CKMR could form the basis for a climate-resilient MP, robust to environmental factors that could affect the indices the current MP relies upon.

How CKMR works: Similar to the use of genetics to identify human relationships, for example a paternity test, CKMR uses the unique genetic signals that any individual gets from its mother and father as a 'tag' shared amongst 'close-kin'; i.e. parent-offspring pairs and siblings. Intuitively, the more kin-pairs are identified in a fixed number of samples analysed, the smaller the population size. Sample sizes needed for CKMR are a function of population size: the bigger the population, the bigger the sample size needed to give statistically precise results.

One of the first applications of CKMR, to Southern Bluefin tuna (SBT) estimated the population size, resolving the most critical uncertainty for the management of the stock and forming the basis for incorporation into subsequent modeling and catch advice. Since 2019, CKMR has been an essential input for the SBT MP. CKMR is also being applied to Pacific bluefin tuna and numerous other tuna, sharks and other species, globally.

Timeline

2024: Study design, sampling feasibility, rough budget and existing sample inventory- completed;
2025: Initiate BFT-E CKMR pilot. Commission decision for additional funding for 2026.
2026: Second field season for sampling and genetic analyses;
2027: Deliver 'pilot CKMR' estimate to inform the MSE review;
2028-2029: Field seasons for sampling and genetic analyses;
2030: Deliver 'Operational BFT CKMR' estimate and options for development of a CKMR-based MP.

3. CKMR modeling.

In order to maximize the chances of a useful outcome for economical expenditure, CKMR studies need to be designed carefully, particularly in terms of overall sample size and its breakdown by year, fishery, etc.; samples from different places can vary a lot in how much information they convey about abundance and other quantities of interest. E-BFT is a particularly complicated case, because of spatial-structure issues associated with multiple spawning grounds within the Mediterranean. The CKMR design process always requires mathematical modeling, based on a realistic understanding of logistic options and some working hypotheses about population size, spatial structure, etc., and computer programming. This process for E-BFT in 2024 has had two phases, summarized in an initial report prior to the Malta workshop in April (Bravington and Fernández 2024a), updated by a supplementary report in July (Bravington and Fernández 2024b). Discussions in Malta clarified some issues around objectives and logistics, including the extent to which existing GBYP samples might be useful; these matters were explored in the supplementary report, together with several alternative designs.

A vast range of sampling designs could be considered. Seven designs were considered in the work done, which were then focused down to three which (noting the caveats below, and in the reports) might be expected to deliver useful results in both the short-term (by 2027, in the hope of having some estimates ready for OM reconditioning) and the medium term (by 2030). Unlike abundance-index surveys, the statistical power of CKMR builds cumulatively, so that the information-content of samples collected early in the program continues to increase as they are compared with more samples collected in subsequent years; thus, CKMR programs tend to become better value-for-money as they are extended in time. The three designs differ in terms of total sample size, and in the breakdown of samples across fisheries. The design with the fewest samples (and hence presumably the cheapest), based on comparing Balearic larvae with samples from different Atlantic fisheries, could give reasonable precision on the estimate of total E-BFT adult population abundance, but there is no way to check its assumptions, in particular the assumption that fish that are originally from different spawning grounds in the Mediterranean are fully mixed in the Atlantic. Adding more samples from different fisheries within the Mediterranean gives the chance to check those assumptions, as the study progresses. The ultimate choice between designs will depend on objectives and on logistic constraints.

It is important to bear in mind that these proposed designs, just like any CKMR design, are conditional on working assumptions about current population abundance and other demographic parameters. Those assumptions have to come from an existing stock assessment, which in the case of E-BFT is highly uncertain, particularly about absolute abundance. The implication is that the number of kin-pairs found after the design has been implemented, and hence the precision of the estimates of abundance, may well differ substantially from the design calculations. If there are substantially more tuna than assumed in the design calculations, then fewer kin-pairs will be found and the precision of estimates will be worse than calculated; conversely, if the true abundance is lower than assumed, then the number of kin-pairs found will be larger and the precision of estimates will be better. Thus -- as with Southern Bluefin Tuna, for example -- adjustments to the sampling design in future may be needed in order to achieve useful precision, based on interim results as the study develops.

The modeling conducted so far is appropriate for design purposes. However, further work would be needed to prepare the model for application of real CKMR data. In particular, the population dynamics model should be extended to incorporate length-structured data from sampled adults and to handle within-cohort larval sibship. Furthermore, the issue of appropriate kinship detection in the presence of within-cohort larval sibship is complex and requires further examination.

4. Sampling protocol, labeling and metadata description and centralized storage facility

4.1 Sampling Protocol

This protocol describes the larval, juvenile and adult sampling necessary for the application of the close-kin mark-recapture (CKMR) methodology to eastern Atlantic bluefin tuna.

This is a large project with a high number of samples collected annually by different institutions and personnel. So, aspects such as coding and labeling of samples, and contamination, are of great importance. A centralized tissue storage facility is necessary to enhance organization, standardize storage methods and labeling, and maintain control over metadata. A centralized facility will enable the presence of a dedicated team for overseeing the preparation and labeling of vials, ensuring subsequent delivery to samplers/institutions, along with the single-use samplers. Since sampling must be divided into several countries, an allocation table must be agreed upon in advance and pre-labeled sample tubes must be sent to the sampling teams well in advance of the harvest season (which varies from country to country). To minimize sampling mistakes and facilitate efficient CKMR sampling, the process should be carried out as quickly as possible while minimizing interference with fish processing at the sampling locations. The possibility of having training courses for the people who are going to do the sampling should be also explored.

4.1.1 Larval sampling

The sampling strategy should be based on collecting larvae at as many stations as possible rather than sampling a few stations with a high abundance of larvae.

High quality (non-degraded) plankton samples preserved in 96% v/v pure ethanol are needed for genetic analyses, and especially for CKMR. In the Mediterranean sampling, it was found that, due to the usually low plankton sample volumes, it is not required a routine change of ethanol after 24hrs from the initial field collection. However, if large plankton biomasses are caught, e.g. with high abundance of phytoplankton, the ethanol should be replaced after 24 hours with new 96% v/v pure ethanol.

Once in the lab, BFT larvae from selected samples should be identified and picked up by trained personnel, storing them in appropriate and correctly labeled vials, and fixed again in pure ethanol 96%. Along the sorting process the larvae should be kept in ethanol to prevent tissue degradation. It is convenient to separate the larvae into jars according to the different larval stages, in order to analyze only those in the pre-flexion stage (reduces the risk of contamination in genetic analysis due to cannibalism).

Metadata for each station includes location (lat, long), gear type and mesh size, station ID, species ID and number of larvae by developmental stage.

4.1.2 Juveniles and adults sampling

Two samples (sample and replicate) shall be collected from each specimen, collecting muscle or fin tissue samples. Depending on the sampler used to collect the tissue, if the sample is large enough, a single original sample can be obtained and split in the storage center to have a replicate. Samples should be fully immersed in the preservation liquid (either 95% ethanol or RNAlater™). Samples should be collected as soon as possible after the fish has been caught.

The sampling team should be preferably composed of two people, one focused on getting the tissue samples and the other in controlling the storage, fixation and labeling of the samples, since in this way the sampling tasks are quicker, easier and less prone to cross-contamination. The person collecting the tissue sample should prioritize keeping the hand holding the handle/tip clean while the other hand can hold the fish.

Actions to prevent cross-contaminations should be applied.

- Ensure sampled fish are properly cleaned before tissue sampling. Cleaning with pressurized water from a hose may serve this purpose.
- Wash hands and use disposable gloves during tissue sampling.
- It is recommended to use single-use samplers. It removes the need for sterilizing equipment (e.g., scissors, knives and forceps) between samples. If this is not possible, forceps and scissors should be washed with clean water and ethanol before each use.
- Avoid mixing of samples. The vials should be labeled clearly according to the agreed format. Samples without proper labels are not valid. The vials should have a unique code (which can be customized with the manufacturer before ordering). The use of the code engraved on the vial saves time for the sampler and avoids errors. Vials should be labeled with a non-dissolving ethanol resistant marker or printed labels or pencil to avoid possible loss of labels.
- Samples stored in ethanol or RNA later can be kept at room temperature, but if cool storage is available onboard it should be used. Once in the laboratory, ethanol levels in the vials should be replaced with equal volume after 24h to ensure sufficient concentration and checked periodically to prevent problems

of evaporation. Storage in the fridge or freezer will reduce ethanol evaporation and is desired. The advantage of ethanol over RNA later is that it is less expensive; however, RNA*later*™ allows easier sample transportation (as it is not flammable and thus does not require special transportation permits).

– Tissue should be fully preserved. Each tissue sample should be placed in individual vials, approximately 20 mg (0,5-1 cm3) of tissue in 1.5 ml of ethanol and ensure that tissue sample is fully submerged.

There are several options for single-use samplers.

1) Gene tag tool, GTT

This tool is used for southern bluefin tuna (SBT), albacore, Spanish mackerel, and a number of other species. For SBT up to 10,000 live fish are sampled; then another 12,000 to 15,000 dead fish are sampled each season. This tool is used to obtain muscle samples. Three elements are needed: handles, biopsy tips and vials with preservative (**Figure 1**).

The handles are a bespoke piece of equipment built by CSIRO. Two handles per sample operation as a minimum. They can take 5.000 samples using the one handle. However, if something goes wrong with that handle, a back-up handle ready to go is needed.

Biopsy tips are single use. The use needs to pack these into a tip box, from where they are used during sampling (hold approx. 100 tips). This allows the user to pick up a tip using the handle without touching the tip itself and risking cross-contamination. The tips are not sterilized prior to use. However, they are only handled while wearing laboratory gloves. To collect two samples per specimen, it is necessary to use two separate tips and two separate vials. The original tip design collects approx. 25 mg of tissue.

Different types of vials can be used from Eppendorf tubes to flat bottom vials. 2 ml Eppendorf tubes can be used (e.g. from Bioplastics www.bioplastics.com). The tissue can remain with the tip in the vial, and the vials can be frozen, later, in the laboratory, the tips are transferred to deep-well dishes and the tissue spun out using a centrifuge. Pre-filled vials can also be used with ethanol or RNALater, store the tip with the tissue in the vial or remove the tissue from the tip. See **Table 1** for cost estimation per sampling unit

2) The LVL genetic sampling tool

This was designed in collaboration with European fisheries scientists and fishing industry organizations (https://lvl-technologies.com/en/). The sampling tool is designed specifically for use on fish and the pointed biopsy tip and rear facing cutting edge enable collection of a c.30mg tissue sample from beneath the skin of the fish being sampled, thus avoiding surface contamination. The three elements necessary for sampling, handles, biopsy tips and tubes (included in racks), are pre-assembled and sterile (**Figure 2**).

This system allows the user to select a sampling tip from a specific tube or vial, collect a tissue sample from the fish and return the tip, with the sample inside, to the collection tube without ever touching the tube or sample directly, thus reducing the possibility of cross-contamination. Barcoded tube racks can be scanned with a SAFE® Tube Rack Reader and sample barcodes imported into databases. See **Table 1** for cost estimation per sampling unit.

During DNA extraction a capper/decapper can be used to remove eight sample tool caps, with samples, from the sample tubes and transfer them to the LVL low profile deep-well plates for the digestion stage of DNA extraction. After a timed digestion, the sampling tools can be returned to the storage tubes for archiving of remaining tissue. This removes the need for sub-sampling of tissue samples thus representing a significant time saving and increasing sample throughput. It also removes any potential of source of contamination by eliminating direct handling of tissue samples **(Figure 3**).

3) Tissue Sampling Unit (TSU)

USA company (Allflex) with representatives in Europe (https://www.biomark.com/tissue-sampling-system/). This sampler is useful for fin clips, a small piece of fin tissue from any fin of a fresh fish, and small sized fish muscle, tissue size should be at a minimum 5 sq. mm.

The Biomark Tissue Sampling Unit (TSU) includes a collection tube (vial), stainless steel blade punch, red plastic blade punch safety clip and is designed for a single, one-time use. The blade punch cuts and pushes the sample directly into the vial automatically sealing upon release. The TSU applicator is used in conjunction with the TSU to collect the sample by cutting the tissue and sealing it into the TSU vial. Allows clean, easy, and efficient collection of tissue samples for DNA analysis without the need for any hand cutting with scissors or use of tweezers (**Figure 4** and **Figure 5**) (https://www.youtube.com/watch?v=Lf3O3WiXD_0).

TSU comes in 96 trays or well boxes which have QR codes and are individually numbered. There are automated systems for reading the bottom of the 96 well box. Each TSU unit comes with a new cutter therefore avoiding cross contamination. The numbering on the TSU can be stored automatically from your reader into an Excel file. See **Table 1** for cost estimation per sampling unit

The manufacturer uses an "in house" developed preservative liquid with no details on the composition of the solution, but they state that the buffer is usable in DNA laboratory settings without restraints on existing DNA extraction protocols and provides a stable medium for multiple extractions. In their web page they state that samples can be conserved up to one year if not frozen, and longer when refrigerated or frozen, but with no specifications of exact time. This is not a problem if samples are going to be analyzed in a short period of time, but for keeping archives of the original samples the solution should need to be changed to RNAlater or ethanol. There is also the option to buy empty vials and fill them with the buffer of interest before sampling. The vials are sealed, so it is also necessary to use TSU Vial Opener Pliers to open them again for extractions.

4.2 Labeling and metadata description

4.2.1 Labeling

The vials should come with a unique code (which can be personalized with the manufacturer before ordering). The use of the code engraved in the vial saves time to the sampler, and avoids many errors that can occur when creating the ID. However, the year (in which it was sampled), the species (BFT), the collector's code and sample identification number must appear on the sample label.

4.2.2 Metadata to be recorded for each tissue sample submitted to approved lab

Fish identification info:

1) Fish ID (add the vial ID)
2) Species (BFT)
3) Tag Info Available (Yes/No)
4) Tag type
5) Tag number info

Sampling data:

1) Sampling Date (in yyyy/mm/dd format)
2) Sampling location
3) Year of capture (year caught dead or alive from the sea)
4) Capture date (if available, date caught dead or alive from the sea in ddd/mm/yyyy format)
5) Capture location area (Areas could be predefined in a map for standardization)
6) Capture Latitude (in decimal degrees if possible, otherwise in 1x1 or 5x5 degree cell)
7) Capture Longitude (in decimal degrees if possible, otherwise in 1x1 or 5x5 degree cell)
8) Fishing Gear (ICCAT codding, BB= baitboat; FAD= fish aggregating device; GN= gillnet; GS= Greenstick; HL= handline; LA= larval survey; LL= longline; MWT= pelagic midwater trawl; PS= purse-seine; PSS= purse seine, small scale; RR= rod-and-reel; TN= trammel net TRAP= trap; TROL= Trolling lines; UNCL= unclassified)
9) From farm origin (Yes/No)
10) Name of Farm
11) Country where the farm is located
12) Collector
13) Collector Email
14) Collector Phone Number
15) Holder, Responsible Party/Institute/Partner (RP), or Principal Investigator (PI)
16) Holder, RP, or PI Email
17) Holder, RP, or PI Phone Number

Biological info:

1) Length (in cm to the nearest cm)
2) Type of length (SFL= straight fork length; CFL= curved fork length; LD1= Straight first dorsal fin length; HeadL= Head length; PreopL= Preopercular length. SL= Snout length; TL= Total length (larvae)). See **Figure 4.6**.
3) Weight (in kg)
4) Type of weight (Round weight (RWT): the weight of the whole fish as it comes out of the water before any processing or dressing; Gutted weight (GWT): the weight of the fish without guts and gonads, but with head, tail (caudal fin) and gills; Gutted and gilled weight (GGWT): the weight of the fish without guts, gills and gonads, but with head and tail; Gutted, gilled and tailed weight (GGTWT): the weight of the fish without guts, gills, gonads and tail, but with head; and Dressed Weight (DWT): the weight of the fish gutted, head off and tail off.)
5) Sex (Visual identification, Male (M), Female (F) or not determined (I))

Other:

1) Comments (on length, on weight, station ID (for larvae only) & other comments)

4.3 Centralized storage facility or tissue bank

As mentioned in the introduction of section 4.1, the BFT Species Group believes that maintaining all samples in a centralized location offers significant advantages, including improved organization, standardizations in storage methods, standardization in labeling and "banking", energy and cold supply assured. etc. The move toward a centralized tissue bank for CKMR also highlights the need for ICCAT to consider developing a tissue bank for all of its biological samples, all ICCAT species, and this is something the SCRS should be considering for recommendations with annual budget implications at this year's annual meeting.

There are currently companies that already provide this type of centralized tissue bank services, and these would be ideal candidates to discuss their ability to take on a larger number of samples. Most tissue banks in Europe are for human and non-human primate tissues. There are also banks for other species, most of them are linked to zoos and the number of samples they hold is very small compared to the previous ones, nevertheless they seem the most feasible option (there is for example one of them, EAZA biobank, which has a relationship with LVL-technologies).

Estimating the cost of a biobank for samples for the application of CKMR to E-BFT is complicated by the aforementioned reasons. **Table 2** shows an estimate of the cost of such a biobank in a scenario in which it is necessary to start from the creation of a facility and, in addition, the annual cost of operation and maintenance of the tissue bank. These figures may vary if it is finally decided to rent the use of a facility already set up, or if it is decided not to take replicates of each sample (if the sampler used takes enough tissue), or if the number of samples per year is lower than contemplated in the budget, for example going from 20-25 000 samples per year in design 0, to only half the number of samples in design 6 (detailed description of sampling designs in Bravington and Fernandez (2024b)), and both designs should include the conservation of GBYP samples already collected in recent years.

4.3.1 Terms of Reference giving a clear description of what is needed for a tissue centralized storage facility

The biobank should receive, curate and maintain the samples, DNAs and associated metadata to be used for the BFT CKMR study.

The biobank should include infrastructure for sample storage, hardware and software for a relational database with associated metadata, and dedicated personnel for ensuring biobank sample pre-processing for storage, maintenance and access.

- The biobank should allow for reception, storage and inventorying of 20-25K samples/year (around 120K samples in total); samples will be stored as fin clips, muscle tissue or larvae (before processing) or as extracted DNA (once processed).
- The biobank should maintain the samples at < -20ºC.
- The biobank should include a laboratory information management system (LIMS), including barcodes that allow sample tracking from collection to analysis.
- The biobank should ensure sufficient and continuous energy supply so that sample quality is not compromised, and database replicate or periodic backups.

Necessary costs (see **Table 2**):

Costs include:

- Infrastructure for sample storage
- Hardware and software for the relational database
- Dedicated personnel for infrastructure maintenance, sample storage (including making replicates when needed, ethanol changes,…), sample inventorying, database maintenance, reporting, respond to sample requests, etc…
- Energy supply
- Consumables

Costs do not include:

- Transportation of samples and custom derived costs if the cost exceeds what is planned (see below)
- DNA extractions

5. Sampling scheme

CKMR requires that the samples from at least one of the considered components of the population, it is adults or juveniles/larvae, be taken in areas where specimens are well mixed, i.e., where catches are made in proportion to the abundance of each subpopulation (Anon 2021, Casas and Saborido-Rey 2023). Taking into account the current knowledge in BFT biology and spatial patterns, as well logistic constraints for sampling high number of individuals by year (in the order of thousands), five areas have been selected as potential sample sources: Balearic sea as sampling area for larvae, Adriatic sea to sample juvenile fishes, taking advantage of Croatian purse seine fisheries, farms located in Western and Central Mediterranean to sample adults captured in the Mediterranean sea by purse seine fisheries and, finally, different fisheries targeting adults operating in the North Atlantic. The estimated number of samples per category has been established based on target coefficients of variation, timeframes and sampling logistics (Bravington and Fernandez 2024a) (**Table 3** and **Figure 7**). Alternative sampling scenarios with smaller numbers of samples have been explored (Bravington and Fernandez 2024b). However, for the purposes of sampling design, the focus in this section is on identifying the most suitable sites and fisheries for sampling. This sampling scheme will be optimized as the CKMR analysis is implemented with real data.

5.1 Larval sampling

Larval sampling will be done in the main Western Mediterranean spawning area, in waters around the Balearic Islands. The planned number of individuals to be sampled by year has been initially set at 8000. This relatively high number has been calculated to account for the fact that the probability of detecting half and full siblings from the same spawning event, i.e., intra-cohort sibship, is high, since larvae originated from the same spawning event may still be schooling together when they are caught. This represents a potential problem, because it would result in redundant information. This number may be reduced if it is demonstrated that a proper selection of sampling stations can minimize the cases of intra-cohort sibship. The assessment of intra-cohort sibship among bluefin tuna larvae sampled within the Balearic Sea is being addressed as an objective in the recent GBYP call for biological studies (Phase 14).

Larval sampling in Balearic Islands is already established and in place as part of the EU data collection program, which funds annually the Balearic larval survey cruise, and hence samples for CKMR can be provided at a minimum cost. The survey platform captures more than enough bluefin tuna larvae each year, but in the end the number of larvae retained for CKMR can easily be scaled up to satisfy the needs of the CKMR sampling if necessary.

5.2 Juvenile and adult fish sampling

The information required for each specimen sampled is detailed in section 4.2, however, it is worth to recall the most important and essential data:

1) Original catch location.
2) The year the fish was caught (removed from the wild population).
3) Date the fish was sampled.

4) Length measurements of the sampled fish.
5) The analysis of the samples will provide the other information that is needed for CKMR:
    a. The age of each fish, this can be obtained by epigenetic aging analysis, and,
    b. Sex of the fish, which can also be determined by genetic analysis.

5.2.1 Juvenile sampling in Croatia

It is proposed to use juveniles caught in June in the Adriatic Sea by Croatia using purse seiners. The number to be sampled annually would be about 2000 individuals. The catches correspond mostly to two year old juveniles. These specimens are transferred to fattening farms where they remain at least 18 months. Therefore, sampling would be done when the specimens are three to five years old. It is not possible to get directly the length at the time of capture for each individual, although length measurements are obtained using stereoscopic cameras during the transfers to the cages. Consequently, the length measure that should be recorded is that taken when the fish is slaughtered, from which the size at capture could be estimated applying available tables of growth in farms.

Sampling can be conducted during the harvest season, which runs from January to the end of February. It has been done previously in farms, including genetic sampling, within the GBYP program. This sampling would need to be substantially increased, but local teams have enough experience and skills to address these sampling needs. Each year 40,000 fish are harvested, which is more than enough to perform the sampling required by CKMR. Since the research team will be directly engaged at the harvest line on the vessels, obtaining fin-clips is more feasible compared to muscle sampling.

5.2.2 Western and Central Mediterranean adult sampling

Fattening farms are the best identified locations for sampling. These farms typically slaughter most of the tuna before the end of the year (average time on farm 4 to 6 months). There are Maltese farms that could easily satisfy the CKMR sampling needs. Maltese farms could not only provide samples from Central Mediterranean, but also from Western Mediterranean, since some of these farms also fatten tuna caught in the waters around the Balearic Islands, although this may vary from year to year. However, in order to ensure W Med sampling, Spanish farms could also be sampled

Other fisheries that could be sampled are the French and Spanish longlines operating in the waters around the Balearic Islands, although the activity of these fleets is very variable and sampling is more difficult to organize.

Biological sampling of several hundreds of individuals by year have been carried out in Maltese and Spanish farms for many years, with sampling being done on processing vessels located in the vicinity of the farms. The process is as follows: The captured fish are slaughtered in the cages, then placed on an intermediate barge that transfers the fish to the processing vessel. On the deck of the processing vessel, before processing begins, length and weight measurements are taken. This deck of the vessel is the best place for sampling prior to processing, as specimens can be measured and/or weighed and there is room for the sampling team. It is also possible and convenient to clean the deck and specimens of blood with a water hose to avoid cross contamination during sampling. In the case of Spanish farms, sampling can be done at the factory once the slaughtered tuna has been landed and before processing. More than 130,000 individuals were processed on Maltese and Spanish farms in 2023, this means that both sampling locations could easily satisfy collecting up to 2,000 adult samples for the Central and Western Mediterranean.

A key issue in using tuna farms as a sampling platform is the ability to distinguish the original location and time of capture of individual fish prior to their transfer to the farm location. If the origin and date of capture of the tuna is unclear because tuna from different fishing operations have been clumped together in a particular cage, that cage should be avoided for sampling. This should be confirmed with the farm prior to any sampling being carried out.

5.2.3 Atlantic adult sampling

Several Atlantic fisheries can provide CKMR samples from adult fish, collecting the 2,000 needed samples. Portugal/Spain/Morocco traps would be the preferred locations, since they can provide high numbers of samples from well mixed populations. Canada + USA existing sampling, carried out within the W-BFT CKMR project, could also provide samples as they include eastern specimens. Japanese longline, France – trawlers/Rod and Reel/longline are other potential sources of well mixed samples, but they could face some logistic limitations to provide high numbers of samples

So, Atlantic Portuguese, Spanish and Moroccan traps are good platforms to collect the needed samples. Tuna are caught when they are entering into the Mediterranean and some traps keep some tuna in captivity for 3-4 months for fattening. Tuna can be sampled after slaughter and when they enter the factory before processing. Head removal is one of the first processing steps, but these heads can be used for genetic sampling and use biometric relationships such as snout length to estimate SFL. The number of fishes caught makes it easy to reach 400 tuna per year by country (1200 specimens).

There are already eastern BFT samples within the Canada and USA W-BFT CKMR sampling project that may provide a good source of samples that have a clear collection method/platform in place. The number of eastern BFT samples being collected each year are approximately 500 in Canada and approximately 700-800 in the USA, these numbers varying each year depending on the proportional composition of the EBFT and WBFT populations in the sampling effort.

Japan currently collects approximately 100-300 samples caught by longline fishery for the GBYP biological studies. The stock of origin of those samples are unknown but most samples were caught in the eastern Atlantic. There is not really a possibility to scale up the current on-board sampling. However, there is an opportunity to scale up sample collections of market fish with additional resources. One issue with sampling market fish is that their tail has already been removed, and hence fork length cannot be measured. So preanal length is measured instead. Other measurements can be made and then fork-length can be estimated using a conversion factor. The number of samples that could be collected through market sampling depends somewhat on human resources. At this time, they are getting about 10-20 samples per month via market sampling.

French fisheries (trawlers, longliners and rod and reel) operating in the Bay of Biscay landed about 330 t of BFT larger than 80 kg (age 7) in 2023, representing about 3,000 individuals in 6 auction markets. Some of those locations are currently opportunistically covered and provide samples to GBYP, and could be of help to CKMR as the current opportunistic sampling could be increased.

Number of two-person teams: 5 for the Mediterranean and 7 for the East Atlantic. Total 12 teams.

6. Genotyping

For CKMR, it is necessary to determine the kinship relationships (e.g. Parent-Offspring-Pair, Half-Sibling) between pairs of individuals. These relationships are inferred based on genetic markers, such as Single Nucleotide Polymorphisms (SNPs). Kinship assignments are based on allele sharing between pairs of individuals and thus, in order to avoid finding alleles shared by chance a large number of loci have to be examined (in the case of SNPs >2,000 are required). Moreover, to avoid any potential biases, these loci have to be neutral. Thus, one of the required steps for CKMR application is to find those genotyping markers, which can be done following alternative procedures: one of them is the reduced-representation DNA sequencing, which allows to cost-effectively finding thousands of SNP markers, as it does not require sequencing whole genomes, in virtually any species, as it does not require any previous genome wide information.

In addition to loci required for kinship, certain other types of "loci" (in a broad sense) also need to be assayed (i.e. measured) for CKMR. These purposes of these additional loci include: sex determination of adults; population genetic discrimination; potentially other loci of biological interest, e.g. genetic inversions; mtDNA haplotype; and epigenetic age estimation. The number of such "loci" is small relative to the number required for kinship, and some can be accommodated at no extra cost during the kinship assay. However, depending on the genotyping technique(s) that are used, some of these "loci" may require separate assays for technical reasons. This is always likely to be the case for epigenetic-age loci, for example; for modeling and logistic (e.g. no otolith) reasons, this will be crucial to have for a proportion of adult BFT CKMR samples. The W-BFT CKMR experience has shown that it is particularly desirable to be able to get age estimates for samples that turn out to be parents, for example, and it is also important to have length-stratified subsamples of age for the population-dynamics component; but the total number of epigenetic age assays will be small compared to the total kinship sample size. The other essential "loci" are for mtDNA haplotype determination, in order to identify maternal vs paternal sibship of larvae. Again, experience with West CKMR has shown that this is a critical data need for models that are able to cope with the high levels of within-cohort sibship found in W-BFT and, from initial analyses, in E-BFT larval samples.

While kinship detection is a rapidly growing field (e.g. within human genealogical research) such an approach is less widely used for fish species. To date there have been several applications to Atlantic bluefin tunas: CSIRO has used Dart-seq for identifying a set of SNP markers for stock assignment and for kin finding McDowell *et al*.

(2022), and AZTI, as part of several GBYP Phases, has used RAD-seq for studying population connectivity and adaptation (Rodriguez-Ezpeleta *et al*. 2019, Díaz-Arce *et al*. 2024a) and for identifying markers for kin finding (Díaz-Arce *et al*. 2024b). From both reduced-representation approaches used, a set of SNP markers for kin finding and other CKMR related needs have been identified and transferred to a sequencing based or an array based assay. Results from both applications indicate the successful identification of kin pairs, both parent off-spring pairs as well as half-siblings, which are the necessary building blocks of a close-kin mark recapture framework.

6.1 Sequencing-based assay

The ongoing CKMR studies for the West Atlantic (2014-present) utilized the genetic data collection approaches developed for southern bluefin tuna, in collaboration with CSIRO (McDowell *et al*. 2022). Initial collections were sequenced using DArT-seq (https://www.diversityarrays.com/services/dartseq/) on larval and adult collections (2016-2017) to identify markers for stock-identification and kinship. A subset of approximately 2700 specific loci markers were selected from the DArT-seq representations for targeted genotyping using DArTcap sequencing (https://www.diversityarrays.com/services/targeted-genotying/). DArTcap data were used to estimate stock-composition, parent-offspring pairs, larval sibship, and id sex of the adult fish caught between 2016 and 2021 for the first phase of the West CKMR project. The sequencing technique for W-BFTt CKMR will change slightly in future to "DartTag", which allows direct incorporation of specific additional markers (e.g. for sex determination, or any other purpose) into the same assay. Epigenetic aging of some adults may also be used in future, and this will require a separate assay. A separate assay has been, and will still be, required for mtDNA (for all larvae, but not adults); the additional mtDNA assay is not expensive.

6.2 Array-based assay

Capitalizing on previous efforts starting in GBYP Phase 5 and to respond to the need of a tool that allows for cost-effective kinship analysis, genetic origin assignment and sex determination, an Axiom Custom Genotyping Array (hereafter called ABFT-Array) has been developed. Briefly, the ABFT array includes a total of 7,000 genomic markers including nuclear neutral markers, used for genetic origin and kinship analyses, nuclear outlier and mitochondrial markers, used for assessing albacore introgression, and sex markers used for sex determination. More details on marker selection can be found in Diaz-Arce & Rodríguez-Ezpeleta (2024b).

The array has been applied to 1920 samples so far, including replicates of some of them, and it has been shown to be robust: 1) it results on a high average genotyping call rate per sample (92%); 2) it results on highly reproducibility even between genotypes derived from different laboratories and between fin and muscle samples derived genotypes (>99.9%); 3) it is not affected by contamination by either providing no result on highly contaminated samples or providing the genotype of the main sample in low contaminated samples.

The array provides the genetic ancestry profile of individuals which can be used to infer origin; obtaining the full ancestry profile and not only a simple genetic stock ID is important in Atlantic Bluefin tuna because due to admixed samples, mostly present in the West, it is not always possible to assign individuals either to West or East. The array contains enough information to identify kins, having already identified more than 20 half-sibling pairs and 3 full-sibling pairs among the more than 250 Gulf of Mexico origin and 1400 Mediterranean origin individuals analyzed so far. The array enables sex determination, allowing the correct sex assignment of 92.6% of the samples to which it has been applied. More details about the performance of the array can be found in Diaz-Arce *et al*. (2024, SCRS_2024_057).

Although the array contains 10 mitochondrial markers, these are most likely not sufficient for determining if two samples share the same mother, which is required in the current CKMR modeling version based on large amounts of larvae (see section 5.1). Currently, testing for the same mother requires mitochondrial genome sequencing, which is expensive and tedious and not viable for tens of thousands of samples. It is currently under evaluation if a set of markers that could be included in a future version of the array could provide the information needed to determine if two individuals share the same mother.

In conclusion, the ABFT-array can confidently monitor the stock mixing and distribution, identify kin pairs at high resolution and determine sex, all at low risk to produce biased results due to sample cross-contamination, which are requirements for application of CKMR.

7. Epigenetic Aging

The estimation of population abundance with acceptable precision using the close kin mark recapture approach relies on the accurate estimation of the age of individuals identified as members of a kin pair, as the year of birth is derived from their age. Recent developments in epigenetics or the use of natural changes show that to obtain age accurately from epigenetic aging it is necessary to calibrate with individuals of known age (Trenkel *et al*. 2022, Petersma *et al*. 2024).

In BFT, age is estimated by counting otolith growth rings. The absolute age obtained by this methodology has been validated by the radiocarbon bomb method (Neilson and Campana 2008) and the strontium: calcium profiles of the otoliths revealed the periodicity of the formation of annual increments (Siskey *et al*. 2016). Recently, the timing of band deposition has been established, which is indispensable for converting band counts into an age estimate (Rodriguez-Marin *et al*. 2022). Direct aging studies have been carried out in the last decade in a coordinated manner among all the laboratories involved in reading the age of this species on both sides of the Atlantic. This means that standardized protocols for age estimation have been followed through several aging workshops and exchanges (Busawon *et al.* 2020, Rodriguez-Marin *et al*. 2020). Readings performed in the different laboratories using the same methodology are therefore comparable, and are considered the "true age", and can be used in the design of an epigenetic clock.

The ICCAT GBYP program funded a pilot study to assess the suitability of epigenetic aging, estimating the age of individuals based on the level of DNA methylation from the analysis of tissue samples (Davies *et al*. 2023). In this pilot study, three hundred and sixty-one samples were successfully processed for methylation scores from samples sourced from the western Atlantic (n=173), Mediterranean Sea (n=177) and Eastern Atlantic (n=11) collections of matching otolith and tissues and decimal age estimated based on ICCAT protocols. The predictive performance was high for the range of epigenetic age calibration models fitted to age and methylation data, with the multiple regression model performing best, with $R^2 > 0.973$ and a mean square error of 0.49 years. The high quality of the fits and the predictive performance of the best performing models suggest that the effects of sex and geographic area on calibrations are unlikely to be substantial. The results from this study show that age estimation using calibrated epigenetic age models and high-quality tissue samples is feasible for Atlantic bluefin tuna and, based on these preliminary results, a single calibration model could be used for samples from both eastern and western populations.

This study recognizes that it is necessary to increase the representation of younger and older age classes to increase the age range over which the clock is useful (Ages older than 17 years are poorly represented). Furthermore, all tissue samples are from muscle tissue, and as methylation patterns have the potential to differ across tissue types, it would also be advisable to conduct a second calibration exercise involving fin-clip tissue samples.

The development of an epigenetic aging clock requires the data to be split into a training and a testing set and Davies *et al*. (2023) applied a five-fold cross-validation approach to compare the performance of various statistical models. In this procedure, data are split into K non-overlapping subsets (K=5 in Davies *et al*. 2023). The relationship is then "trained" with four of these subsets and tested with the fifth. The procedure is then repeated using another set of four subsets as a testing set. This allows an examination of model performance with data not used in the development of the model. The 5-fold cross validation approach essentially means Davies *et al.* (2023) used an 80/20 split but with 5 different testing sets.

The cost per individual is likely to be a function of the number of CpG sites ultimately chosen for routine epigenetic aging. Epigenetic age estimation requires molecular laboratory expertise, but does not require species-specific knowledge of otolith reading. Reading the age of otoliths from long-lived species such as BFT, on the other hand, is laborious and requires species-specific expertise, which takes time to acquire.

8. Kinship analysis and abundance estimation

8.1 Kinship analysis

After the genotyping of samples collected at different locations has been done, genotypes identified as having an Atlantic origin need to be separated before proceeding with the kinship analysis. Pairwise kinship comparisons involve checking for parent-offspring pairs (POPs) and half-sibling pairs (HSP) in all possible admissible pairs across locations and time. Intra-cohort juvenile comparisons are excluded to avoid biases derived from poor mixing that would lead to an excessive number of HSP, and in turn to underestimated population estimates.

When sampling young juveniles from the same cohort (e.g., larvae), there is a possibility that litters from the same spawning event are caught together resulting in a disproportionate number of full-siblings and/or half-siblings in the sample. Even if the collection of larvae is spread out over many sets to reduce the problem, an analysis of within-cohort sibship needs to be conducted to account for this lack of independence and avoid overestimating the precision of abundance estimates. The new approaches and software recently developed for the Western BFT (McDowell *et al.* 2022) will be applied initially to perform a sibship analysis of close to 3800 larvae collected during the 2022 ichthyoplankton Balearic survey, which will be genotyped during 2024 (GBYP Biological Studies Phase 14).

In general, the level of kinship in each pairwise comparison is quantified using a specific statistic to discriminate between kin and non-kin. A threshold for classification is set so that the expected number of false positives from unrelated pairs is very small. The probability of false negatives (i.e. HSPs with a statistical value lower than the threshold), on the other hand, needs to be calculated based on the mean and variance of the statistic for HSPs to correct for bias.

Kinship analyses have been performed in 1379 Eastern ABFT samples genotyped with the ABFT-array, including larvae, adults, and YOY. 1 FSP (between Mediterranean larvae from the same year) and 11 HSP have been found (5 between Mediterranean larvae from the same year and 6 between adults from feeding aggregates).

8.2 Abundance estimation

The CK data used for estimating abundance, and other population dynamics parameters, are the number of POPs and HSPs encountered given a total number of pairwise comparisons made for the different categories of sampling locations and periods. The probability of finding a kin in a given comparison is a function of the times and locations of capture, and the sex and age (or size) of the individuals compared, together with the population dynamics parameters to be estimated (i.e., number of births from each spawning location over time, mixing parameters, size-specific fecundities, and natural and fishing mortality parameters). These probabilities are specified in Bravington and Fernández (2024a) and were developed specifically for Eastern Atlantic bluefin tuna, assuming three spawning stocks within the Mediterranean.

The population dynamics model used for evaluating sampling designs (Bravington and Fernández 2024b) provides a good starting point for developing the model that will be used for actual estimation, but some non-trivial modifications need to be introduced. As explained in Bravington and Fernández (2024a), some simplified model assumptions were made, especially about fishing mortalities and recruitments, which were considered adequate for the design stage. However, for a stand-alone estimator of abundance, some of these assumptions need to be revised, requiring the use of catch data to inform the estimation of fishing mortalities and recruitments. In addition, the model used for the design assumed that the age of all genotyped individuals was known; in reality, not every fish will be aged, and therefore the model needs to be expanded to incorporate the uncertainty in the age of fish that are only measured. The specific formulation of the estimation model needs to be defined and implemented, including specification of the likelihoods for the different data components. The possibility to discriminate among alternative assumptions, especially about mixing of spawning components, will depend on the sampling design and data availability.

9. Project management and current status, cost estimation and funding

9.1 Project management and current status.

The design of a CKMR project requires knowledge of the biology of the species, its spatial structure and approximate estimates of the size of the spawning populations. In the case of E-BFT, assumptions had to be made because, despite the numerous studies on this species, there is uncertainty on aspects such as, to name a few, the spawning stock biomass (evidenced in the last stock assessment) or the spawning fidelity in the different spawning areas of the Mediterranean Sea. These aspects are important because in order to obtain a reasonably precise estimate of abundance, sampling must be done in proportion to the population size and part of the sampling in at least one component, larvae/juveniles or adults, must be done in areas with well-mixed populations.

Therefore, setting up a CKMR project for E-BFT requires extensive planning because, although the sampling design is logistically feasible from a small number of selected fisheries (and a larval survey), there are numerous elements that need to be coordinated. These include: purchase of sampling material, coordination of sampling teams, coordination of sample shipment, tissue bank follow-up. Coordination is also needed between the modeling, genomic analysis and epigenetic analysis teams, and finally, the financial management of the entire project. This requires dedicated project management. As in other ICCAT endeavors of this magnitude this may be most

effectively accomplished by a dedicated staff who can focus on the project coordination during its lifespan and who would be working under the umbrella of the GBYP. The current GBYP coordinator is the person with the right profile and could carry out this important task of coordinating the E-BFT CKMR.

In the following, we will look at the state of development of the different elements necessary for the implementation of the CKMR in the E-BFT:

1) CKMR Modeling.
   - *Done*. A breakthrough in the modelling of CKMR for application in E-BFT has been made, allowing different sampling scenarios to be evaluated.
   - *Ongoing.*Further work would be needed to prepare the model for application of real CKMR data. In particular, the population dynamics model should be extended to incorporate length-structured data from sampled adults and to handle within-cohort larval sibship. It is also recommended that the first kinship analyses are carried out by the modelling team.
2) Sampling.
   - *Done.* Sampling protocols are in place and sampling possibilities in the selected fisheries/campaigns have been analysed and are feasible. Useful samples from both Atlantic stocks that have been collected in recent years are also available. The needs and estimated costs of a tissue bank have been identified.
   - *Ongoing.* A decision needs to be made regarding the selection of a sampling scenario from those proposed in the CKMR modelling study. It is also necessary to select a single-use sampler. And very importantly, a tissue bank needs to be established.
3) Genomic analysis
   - *Done.* Both genotyping approaches used for BFT CKMR are working, SNP-array for E-BFT and DArTcap for W-BFT. Both methods satisfactorily identify stock ID, sex, and kinships. There is a GBYP phase 14 project to evaluate kinship among bluefin tuna larvae sampled within the Balearic Sea campaign. And work is ongoing to identify the mitochondrial markers needed to address larval sibship due to high levels of within-cohort sibship.
   - *Ongoing.* It is necessary to include, in both genotyping platforms, mitochondrial markers to identify maternal versus paternal sibship of larvae.
4) Epigenetic analysis
   - *Done.* A pilot study has demonstrated that epigenetic ageing is feasible for Atlantic bluefin tuna and is likely to be more cost-effective than ages obtained through conventional otolith ageing, particularly in the context of the sampling (and sample sizes) required for CKMR.
   - *Ongoing.* Muscle tissue has been used in this pilot study, and as methylation patterns have the potential to differ between tissue types, it would also be advisable to perform a second calibration exercise with fin-clip tissue samples (it is not always possible to obtain muscle samples). This methodology is in the research and development phase, and needs to be patented and licensed to a genomic analysis company for routine analysis at a reasonable cost.
5) Funding
   - *Done.* A preliminary cost estimate has been made.
   - *Ongoing.* It is necessary to see what sources of funding are available. The choice of sampling design will determine the costs**.**

9.2 Cost estimation and funding

An estimate of the costs of implementing the CKMR for E-BFT has been made. This cost estimate includes modeling, sampling (including tissue bank), analysis and personnel costs for coordination. These estimates have been made using the sampling design considered the most favorable, in view of the number of samples and the lower CV estimates for SSB, in Bravington and Fernandez (2024b) (**Table 4**). These represent only approximate costs as the actual costs will depend upon what external parties actually put into calls for tenders.
This cost estimate does not include the payment of the people doing the sampling. Most of the sampling is for EU fisheries. Currently, the sampling of fisheries is done in this CPC financed by the European Fund industry, fisheries and Aquaculture (FEMPA). BFT sampling will need to be amended to meet new requirements for CKMR.

The larval survey in the Balearic Islands is a campaign already funded by the EU to obtain an index of relative abundance of spawners. The larvae collection is covered by this campaign, the added cost would only consist of sample preparation, which is included in the cost estimate for sampling (**Table 4**).

Another possible way to encourage sampling in fisheries facilities or fattening farms, can be addressed by labeling tuna products that contribute to research. A similar approach has been used in southern bluefin tuna (SBT), where the processing of tuna heads due to otolith sampling is identified as contributing to research on the resource. This has resulted in a cooperative attitude and significant contributions from the fishing or processing sector.

A budget estimation was performed using the designs with different numbers of samples (design 0 with 16000 new samples vs design X with 8000 new samples) to see the influence of sampling with replicates. Obviously, sampling with replicates entails an extra expense in sampling units and tissue bank, as well as in sampling time. The cost overrun, without considering this last factor, can be up to 5-8 % (**Table 5**). It seems therefore advisable to perform the sampling by collecting only one sample and to perform a replicate of this sample in the tissue bank, if necessary. In order to apply this procedure, it is necessary that the single-use sampler obtains a sufficient amount of sample to be able to make a replicate. This procedure also reduces the possibility of sampling errors and simplifies the coding of the sample vials.

One option for funding within existing resources would be to move some GBYP objectives to an alternate year schedule, which would provide this funding in alternate years. There is also an option that the Commission could consider which would be a specific scientific quota allocation of up to 100 mt which could be auctioned off, external to any CPC quota, and fund the CKMR project. Hence the science that supports the fishery could be self-funded by the fishery itself.

Projects of similar magnitude are underway in other ocean basins so while the scale appears high, it is not outside of the realm of feasibility.

10. Future integration with existing close-kin development work (both EBFT and WBFT CKMR)

EBFT CKMR benefits from much of the groundwork conducted for southern bluefin tuna (Bravington *et al*. 2016), western atlantic bluefin tuna (McDowell *et al*. 2022) and the investment of GBYP in developing genotyping technology (Diaz-Arce & Rodríguez-Ezpeleta 2024) making this a possibility. One of the benefits realized by CCSBT is that while it was originally conceived as a single point estimate of abundance, CKMR and gene-tagging now provide a time series of genomic-based abundance estimates that are fully incorporated into the stock assessment and the management procedure. Having a unified, pan-Atlantic genomic based approach could serve Atlantic bluefin tuna similarly in the near-future. For example, WBFT will have an 8-year time series of CKMR abundance by 2027 that could possibly be considered as a genomic-based index to drive an empirical management procedure.

Options for integration of EBFT and WBFT CKMR data collection programs include EBFT using the same genotyping method and markers currently used for WBFT or WBFT using the same genotyping method and markers currently used for EBFT. In either scenario, archived samples previously genotyped by one method could be re-genotyped so as to be integrated into a unified analysis. Benefits of such integration would be a greater ability to understand mixing and potential evolutionary changes in the populations, in addition to some future cost-savings in avoiding having to re-genotype samples of eastern origin collected in western Atlantic fisheries, for example in Canada.

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Table 1. Cost per type of single-use samplers (€).

	Handle / Sampling tool / Applicator	Biopsy tips or tissue collection cap & vials (unit)
Gene tag tool GTT	450	1,72
LVL genetic sampling tool	-	1,20
Tissue Sampling Unit TSU	62,5	1,8
Average cost	256,25	1,57

Table 2. Approximate budget in relation to tissue bank.

ITEM	*TOTAL COST*
Sample Storage Freezers and storage racks (1)	58,800 €
Initial investment in software – personnel (1)	24,000 €
TOTAL INITIAL INVESTMENT	*82,800 €*
Energy supply/year	4,800 €
Maintenance of sample & DNAs and associated metadata/year - personnel (2)	64,000 €
Consumables & Reagents/year (2)	2,000 €
Customs and Transport/year (3)	8,000 €
TOTAL/YEAR	*78,800 €*

(1) Initial investment, assuming storage for an approximate amount of 100,000 samples. (2) Assuming 20,000 samples per year. (3) assuming 20 shipments per year

Table 3. Number of samples per year in the initial sampling scheme for CKMR. (Table 4 from Bravington and Fernandez, 2024 SCRS/2024/053)

Number of samples per year					
	Larval survey	Juvenile fishery	Adult fisheries		
	Balearics: $Wlar$	Croatia: $CROjuv$	West Med: Wad	Central Med: Cad	Atlantic: $ATLad$
2019-2024	3000 (excluding 2021)	0	0	0	0
2025-2030	8000	2000	2000	2000	2000

Table 4. Budget estimation for the sampling design number 5 considered in Bravington and Fernandez (2024b).

Budget	*Pilot CKMR*			*Operational CKMR*		
	2025	*2026*	*2027*	*2028*	*2029*	*2030*
CKMR pilot studies (epigenetics & mtDNA) (1)	€ 140.000					
Genotyping and bioinformatics (2)		€ 1.198.000	€ 191.000	€ 336.000	€ 266.000	€ 406.000
CKMR modeling	€ 50.000	€ 25.000	€ 25.000	€ 50.000		€ 30.000
Sampling collection (Larvae, Juveniles, Atl. Adults), processing and curation and tissue bank	€ 145.000	€ 218.000	€ 129.000	€ 129.000	€ 129.000	€ 129.000
Program coordination	€ 235.000	€ 200.000	€ 200.000	€ 200.000	€ 200.000	€ 200.000
TOTAL (3)	€ 570.000	€ 1.641.000	€ 545.000	€ 715.000	€ 595.000	€ 765.000

(1) Pilot studies will address use of mtDNA and epigenetic aging
(2) Assumes that mtDNA and epigentic aging costs will be reduced in half during operational phase, either through gains in efficiency or improved modeling, sample size is the same (10.792) but the overall costs are spread over different years.
(3) Total does not account for inflation and values are rough approximations. 2006 includes the cost of analysis of historical samples.

Table 5. Budget estimation using designs with different numbers of samples to see the influence on the cost of sampling using replicates (€).

	Design 0 (16000 new samples)	**Design X** (8000 new samples)
	1rst year (1)	1rst year (1)
With replicates	659000	411000
Without replic.	606000	383000
Sampling teams costs are not included		
(1) Includes extra work for the completion of the CKMR modelling and attending to the effect of sibship of larvae. It also includes the initial investment for the tissue bank.		

Figure 1. Left: tips loaded into boxes. Centre: Tip picked up by handle. Right: Tip with tissue dispensed into vial. Pictures from Russell Bradford – CSIRO

Figure 2. Handle, biopsy tips and tube racks (Pictures from LVL, https://lvl-technologies.com/en/).

Figure 3. Capper/decapper can be used to remove eight sample tool tips (Pictures from LVL, https://lvl-technologies.com/en/)

Figure 4. Allflex/Biomark TSU applicator, stainless steel blade punch with red plastic blade punch safety clip and TSU Trays (96) (Pictures from https://www.biomark.com/tissue-sampling-system/).

Figure 5. Use of the TSU applicator on the pectoral fin of an ABFT (picture by IFREMER).

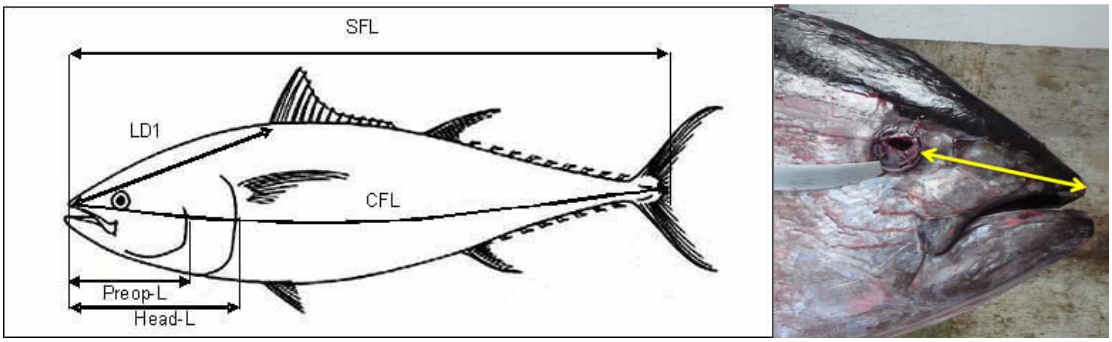

Figure 6. Types of measurements of Atlantic bluefin tuna: Straight fork length (SFL), Straight first dorsal fin length (LD1), Curved fork length (CFL), Head length (HeadL) and Preopercular length (PreopL) and Snout length (SL) (Figures from Secor *et al*. (2014), Rodriguez-Marin *et al*. (2015).

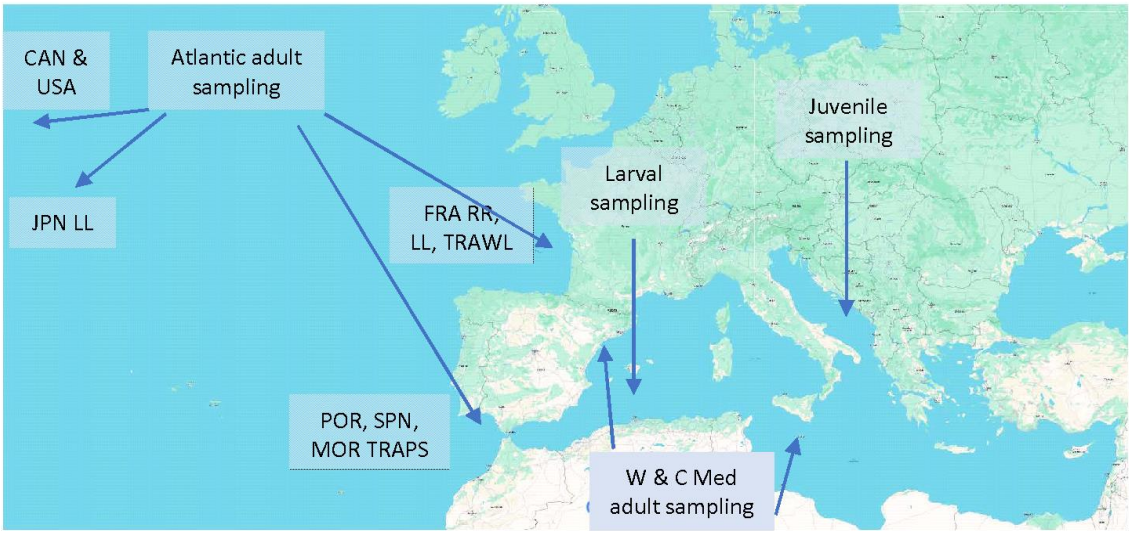

Figure 7. Location of samples to apply the CKMR methodology to E-BFT