REPORT OF THE 2023 ICCAT GBYP WORKSHOP ON BLUEFIN TUNA CLOSE-KIN MARK-RECAPTURE, INCLUDING BIOLOGICAL SAMPLING COORDINATION

(hybrid/Madrid, 14-16 March 2023)

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SUMMARY

The GBYP Workshop on Bluefin Tuna Close-Kin Mark Recapture (CKMR) focused on the analysis of relevant factors for the implementation of the approach in the eastern Atlantic bluefin tuna stock, with the goal of presenting a feasibility study, including a workplan with cost estimations, to the SCRS in 2024. The requirements for CKMR and the current knowledge of Atlantic bluefin tuna (ABFT) reproduction and population structure were reviewed, and examples of applications of CKMR methodologies in other fish species were provided. The genetic studies carried out to date for ABFT stock identification, kinship analyses, sex determination and epigenetic ageing, were summarized and discussed. It was proposed that a comparison be made between the two methodological approaches applied thus far for kinship determination in ABFT, i.e., the one applied in the ongoing western stock CKMR study and the one developed under the GBYP program, and that the possibility of including alternative techniques be explored. Sampling opportunities for eastern ABFT stock CKMR implementation were discussed. Finally, a list of recommendations for future steps and a tentative timeline for their implementation was elaborated.

RÉSUMÉ

L'atelier du GBYP sur le marquage-recapture de spécimens étroitement apparentés (CKMR) de thon rouge a porté sur l'analyse des facteurs pertinents pour la mise en œuvre de cette approche dans le stock de thon rouge de l'Atlantique Est, en vue de présenter, au SCRS en 2024, une étude de faisabilité, incluant un programme de travail et des estimations des coûts. Les exigences pour CKMR et les connaissances actuelles sur la reproduction et la structure de la population de thon rouge de l'Atlantique (ABFT) ont été examinées et des exemples d'application des méthodologies CKMR à d'autres espèces de poissons ont été fournis. Les études génétiques menées jusqu'à présent pour l'identification des stocks d'ABFT, les analyses de parenté, la détermination du sexe et la détermination de l'âge épigénétique ont été résumées et discutées. Il a été proposé d'établir une comparaison entre les deux approches méthodologiques appliquées jusqu'à présent pour la détermination de la parenté pour l'ABFT, c.-à-d. celle appliquée dans l'étude CKMR en cours pour le stock de l'Ouest et celle développée dans le cadre du programme GBYP, et d'envisager la possibilité d'inclure des techniques alternatives. Les possibilités d'échantillonnage pour la mise en œuvre de CKMR pour le stock de l'Est d'ABFT ont été discutées. Finalement, une liste de recommandations concernant les futures étapes et un calendrier provisoire pour leur mise en œuvre ont été élaborés.

RESUMEN

El taller del GBYP sobre colocación y recuperación de marcas en atunes rojos estrechamente emparentados (CKMR),se centró en el análisis de los factores relevantes para aplicar el enfoque en el stock de atún rojo del Atlántico oriental, con el objetivo de presentar al SCRS en 2024 un estudio de viabilidad que incluyera un plan de trabajo con estimaciones de costes. Se revisaron los requisitos de CKMR y los conocimientos actuales sobre la reproducción y la estructura del stock del atún rojo del Atlántico (ABFT), y se ofrecieron ejemplos de aplicaciones de las metodologías de CKMR en otras especies de peces. Se resumieron y debatieron los estudios genéticos realizados hasta la fecha para identificar el stock del atún rojo del Atlántico (ABFT), llevar a cabo análisis de parentesco, determinar el sexo y la edad epigenética. Se propuso realizar una comparación entre los dos enfoques metodológicos aplicados hasta ahora para la determinación del parentesco en ABFT, es decir, el aplicado en el estudio en curso de CKMR del stock occidental y el desarrollado en el marco del programa GBYP, y estudiar la posibilidad de incluir técnicas alternativas. Se debatieron las oportunidades de muestreo para la aplicación de la CKMR en el stock oriental del ABFT. Por último, se elaboró una lista de recomendaciones sobre los futuros pasos a seguir y un calendario provisional para su aplicación.

KEYWORDS

Close-kin mark–recapture (CKMR), Atlantic Bluefin tuna, genetic methodologies, stock structure, epigenetic ageing

1. Opening, adoption of agenda and meeting arrangements.

The workshop was held in a hybrid format at the ICCAT Secretariat in Madrid and online, from 14 to 16 March 2023. Dr. Enrique Rodríguez-Marín (EU-Spain) and Dr. John Walter (USA), the Rapporteurs for the eastern Atlantic and Mediterranean, and western Atlantic bluefin tuna stocks, respectively, opened the meeting and served as Co-Chairs. They described the objectives of the workshop, which were to analyze the relevant factors for developing a feasibility study for Close-Kin Mark Recapture (CKMR) based abundance estimates for eastern Atlantic bluefin tuna (E-ABFT), and to start planning a sampling design. This feasibility study will be developed during 2023 and 2024 with the goal of presenting a proposal to the SCRS in 2024. Dr. Mauricio Ortiz, on behalf of the ICCAT Executive Secretary, and the GBYP coordinator, Dr. Francisco Alemany, welcomed the participants. The Chairs proceeded to review the agenda, which was adopted with no changes (**Appendix 1**). Dr. Erin McClelland from GBYP staff served as the rapporteur of the workshop report, assisted for each of the agenda items by workshop attendees as identified at the beginning of each section. The List of Participants is included as **Appendix 2**.

2. Brief overview of CKMR

SCRS/P/2023/019 Close-Kin Mark-Recapture and Eastern Atlantic Bluefin Tuna

The invited expert, Dr. Mark Bravington, provided an introductory presentation describing the main ideas behind Close-Kin Mark-Recapture (CKMR), with particular focus on features relevant to Atlantic bluefin tuna (ABFT).

The basic idea behind CKMR is that each young fish genetically marks its mother and father at the time it is born. Genetic analysis of tissue samples, e.g., those taken from fishery catches, then permits the identification of "recaptured" individuals from those genetically marked. Statistical methods can then be used to provide estimates of absolute adult population abundance (or standing stock biomass (SSB)) as well as information on other relevant population parameters (in particular, relative fecundity at size/age, fishing mortality (Z) and possibly natural mortality (M), population connectivity, etc.). The methodology can therefore provide core stock assessment information and reveal considerable biological information without the need for fishery CPUE or fishery-independent surveys.

For CKMR, two types of close kin are particularly relevant: Parent-Offspring Pairs (POPs) and Cross-Cohort Half-Sibling Pairs (XHSPs). The genetic samples are all compared with each other to identify these kin pairs. Most of the comparisons will give an "Unrelated" result, but a few comparisons will reveal kin pairs. The probability that one of these comparisons results in a kin pair depends on the abundance of reproducing individuals in the population and certain properties of the animals, e.g., their ages, if one of them was alive and mature when the other one was born, etc. The formulae for these probabilities are case-specific and can be embedded as a likelihood in a stock assessment-type model.

CKMR is very informative and can provide information about absolute abundance, but it requires careful engineering. Five key items were identified as needed for a successful project: 1) development of an appropriate CKMR statistical model; 2) designing an appropriate sampling scheme (i.e., where to sample, what types of samples, how many sample, etc.); 3) implementation of the field sampling correctly (i.e., avoiding contamination, measuring what is needed, etc.); 4) accurate genotyping; and 5) identification of kin pairs. Multiple cohorts of juveniles, with good age information, should be sampled to provide information on both adult abundance and survival; good age information, as well as the full size/age range of adults and sex information are also needed. Sample age information is important for CKMR. Epigenetic aging (using the same tissue sample as that used for CKMR) seems to be a very promising and cost-effective approach for aging bluefin tuna. It is also crucial to collect enough samples to be able to obtain a suitable number of recaptures of both POPs and XHSPs, otherwise the procedure will not provide adequate information to estimate abundance or survival. The sampling program must be a multiyear exercise, as it takes a few years to have enough samples to develop basic estimates and to confirm or refute hypotheses made during the design phase (e.g., on population structure and connectivity). Results from the first sampling phase can provide insight into the number of samples required for a robust estimation of

population size based on observed rates of recaptures (for POPs or XHSPs). As a starting point, it was suggested that at least 50-100 kin pairs are needed in order to obtain estimates with reasonable precision. The number of samples required to find that many kin pairs depends on the total adult population size, as well as on other aspects of the demography and the sampling program. Therefore, a key part of the design phase is to plan the sample size and composition based on available information (e.g., a provisional stock assessment).

Once a result is obtained, it might be decided to stop the CKMR work (e.g., if the objective was to ground truth a stock assessment), but it might also be decided to continue with the CKMR program (as has occurred with southern bluefin tuna, SBT) in order to update estimates of stock abundance, to improve knowledge (e.g., connectivity, mixing, natural mortality), or for use in the development of genomics-informed management procedures. The main difficulty for CKMR is getting the design right and implementing an appropriate sampling scheme. Once that is in place, continuing the process is much simpler.

In past CKMR projects for other stocks, the greatest problems have arisen from bad length/age measurements and/or inadequate planning. SBT was very successful in its implementation, with the main reasons for its success identified as being that there was very careful planning and complete control of sampling (i.e. high quality tissue samples, accurate length data full-size range of adults, and juveniles of known age based on their length). This was for a spatially simple system comprised of one stock with a single identified spawning area, and with a sizeable team of people with well-defined roles covering the full range of skills needed.

A major problem in CKMR occurs if there is correlation between the sampling and breeding probabilities that is not addressed in the model. This is the CKMR analogue of "unmodelled heterogeneity of capture probability" in classical mark-recapture and, as in that case, can lead to bias if overlooked. Spatial population structure, whether heritable or not, is a potential source of this kind of problem (e.g., sampling adults on one spawning ground and juveniles on a different one). Although such correlations cannot always be foreseen or avoided, it is sometimes possible to eliminate bias through careful sampling and appropriate modelling, at a minimum with the aim of being able to detect any substantial violation of assumptions.

In the discussion of this presentation, the question was asked if/how CKMR can deal with the fact that not all mature tuna succeed equally at spawning, i.e., if CKMR can distinguish between a situation with a small number of very successful spawners and one with a large number of less successful spawners, and what happens in relation to unsuccessful spawners. The presenter responded that (i) systematic variations in reproductive success that might be linked to adult sampling probability, e.g. due to adult body size, should already be taken into account in the probability formulae for each case, and (ii) additional random variation ("luck") in reproductive success in any given year is not a problem because the core CKMR assumption remains true regardless, i.e., that each juvenile had exactly 1 mother and 1 father. There is no requirement nor assumption about how many surviving offspring each adult had. For Half-Sibling Pairs across cohorts (XHSPs), any systematic effects (e.g., adult body size) do need to be taken into account in the model to avoid potential biases as per the preceding paragraph, although additional random variation in luck across two separate spawning events does not matter. As a footnote, in some circumstances the incidence of same-cohort siblings can provide information on the extent of "reproductive luck", but that is basically a "byproduct" of CKMR and is not essential for estimating the most important demographic parameters.

The presentation then introduced spatial aspects relevant to CKMR, focusing on populations with discrete breeding and sampling sites, as is known to occur for ABFT. Useful concepts to keep in mind are:

- Assignable: an individual's breeding site can be inferred from its genetics
- Heritable: breeding site not distinguishable from genetics, but acquired from parents
- Faithful: returning to the same breeding site year after year, not necessarily the individual's natal site
- Sticky: faithful generally, but sometimes may change
- Random: breeding changes independently from year to year

If the term "subpopulation" is used to refer to "adults that repeatedly use a specific breeding site", then the sampling sites may be:

- Well-mixed: the proportion of animals that use that site is the same for all subpopulations, i.e., the relative abundance of each subpopulation at that site is equal to its relative abundance in the overall population
- Pure: the site is only used by only one subpopulation
- Mixed: any case intermediate between the previous two

The general formula for spatial CKMR was given using POPs rather than XHSPs, as this is the more general case. CKMR is used for calculating the probability that A (a potential parent) is the actual parent of J (a potential offspring; usually but not necessarily immature), which requires that A was alive and mature and in the same spatial region where J was born at the time of that birth. Several conceptual examples were shown of the implications that the spatial structure, population behaviour (i.e., heritable, faithful, etc.) and type of mixing in the sampling sites (well-mixed, pure, etc.) may have for the CKMR probabilities.

Sampling for CKMR does not need to happen everywhere that the species is fished, even when there is some spatial structure. In fact, as long as there is one sampling site where either potential offspring (usually juveniles), or potential parents (adults), are well-mixed, then in principle it does not matter where the other samples are taken, even if those other locations are not expected to be well-mixed. However, to verify the assumption that one site really is well-mixed, it may be necessary to sample in several additional sites. Multi-site sampling may also be necessary in order to resolve the nature of spatial structure (in terms of the definitions above), to estimate the degree of mixing in specific sampling sites, and to fully estimate all important demographic parameters. Although it is important for CKMR design purposes to have some prior ideas about how spatial structure (if any) might work for a species, it is not essential to understand every detail in advance, because CKMR data themselves are very informative about connectivity and mixing. For example, if comparisons are made between Norway adults versus Balearic larvae, and comparisons are also made between the same Norway adults versus young fish from Croatia, then if Norway is well-mixed the same rates of POPs should be seen in the comparisons from Balearic larvae or from young Croatian fish.

It should be theoretically possible to specify a valid CKMR model (i.e., not subject to any "unmodelled heterogeneity" bias as described above) to deal with a specific hypothesis about spatial structure for a proposed set of sampling sites. However, that does not necessarily imply that there will be enough information in an actual CKMR dataset to estimate all the parameters of that model, nor perhaps to discriminate between hypotheses (e.g., about whether or not sampling sites are well-mixed, or whether breeding sites are heritable). The ability to achieve such estimation depends on having an adequate sampling design, in particular with adequate sample sizes per location. For some species with very intricate spatial and/or social structures, it might never be possible to sample adequately but for EABT the situation does not seem impossibly complex. Dr Bravington commented that, for a situation like EABT, a small number of well-chosen sites with high sample sizes would be much better for CKMR than a large number of sites with small sample sizes. This is because each new site will lead to extra parameters to estimate (concerning mixing proportions) and extra sub-hypotheses to consider, but without enough site-specific data to support the complexity. Fully resolving all spatial complexities will take time, even with the power of CKMR; thus, a reasonable strategy might be to start with a fairly simple hypothesis about spatial structure, within which the number of parameters would be small enough to allow reliable estimation, and to sample in such a way that any major departures from that hypothesis will eventually become clear in the data (e.g., by detecting different POP rates as in the example at the end of the previous paragraph).

With these general ideas in mind, several initial ideas for potential sampling of EABT were considered, assuming three main breeding sites in the Mediterranean (Balearic B; Central C; "everything else" E) together with the possibility of site-faithful breeding. Further examples and discussion of possible sample strategies are presented in section 11 below.

Some participants made comments to be taken into consideration:

- Balearic fish are taken mainly to farms in the Spanish coast.
- Maltese farms will include fish from a wide area, mainly from the Central Mediterranean, but also from further east.
- The Central Mediterranean will be a more mixed area for adults, including both migrants and "residents", possibly in different proportions each year. Part of the population may be from the western side of the Mediterranean and part from the eastern side.
- The bulk of the fisheries, historically, are from the Central Mediterranean. A certain proportion of the stock stays in the Mediterranean for longer than 1 year, but it is not known for how long they stay (or if they even leave the Mediterranean), and what proportion of the population that represents.
- A suggestion was made to focus on Mediterranean and GOM mixing in the Atlantic, and how CKMR is complicated by this issue. It was questioned whether this would be a bigger problem for CKMR for the Western BFT stock than for the Eastern BFT stock. The presenter indicated that he does not regard the need to separate samples into Eastern/Western stock of origin as a real problem, because it is easy to assign each sample to Eastern/Western origin genetically (and then to use only the fish with high assignment probabilities). Therefore, it would be possible to use samples from anywhere in the Atlantic,

if that would help to get well-mixed samples for older fish. The matter of the "mixed" component of both stocks in the Slope Sea was also pointed out as a further complication for carrying out two separate CKMR projects, while a single project would possibly address this aspect.

Several of these aspects are discussed in later sections of this report, and many of the issues need to be examined during the design phase of the CKMR project, including implications for potential biases.

3. Relevant case studies

Three case studies of CKMR on bluefin tuna were presented, providing an overview of the sampling design and methods developed for Southern bluefin tuna, western Atlantic bluefin tuna (WBFT), and Pacific bluefin tuna (PBT). Southern bluefin tuna represents the first application of CKMR and has been ongoing since 2006. PBT and WBFT studies were implemented in 2015 and 2016 respectively, and sampling efforts have demonstrated the feasibility of CKMR, in particular the ability of genomic approaches to identify sibling and parent-offspring relationships. Overall, the presentations provided a broad demonstration of the utility of CKMR for estimating abundance for bluefin tuna populations across a wide range of study systems and different life histories.

3.1 Southern Bluefin Tuna CKMR

SCRS/P/2023/025 Key considerations and lessons learnt from the implementation of CKMR for SBT: 2006-2023

SCRS/P/2023/025 presented the key considerations and lessons learned from the implementation of CKMR for southern bluefin tuna. The main lessons included the following: 1) the value of starting the sampling sooner rather than later (preferably the collection of thousands of juveniles and adults from selected locations); 2) that time spent on good design is time well spent; 3) the importance of stakeholder engagement early in the process; 4) the recommendation to leave the genetics to the experts; 5) to focus on relevant analogies with which non-geneticist are familiar; 6) emphasizing the strengths of the approach; and 7) the benefit of being open about the assumptions. Additional details on genetic approaches, parent-offspring pair analysis, integration of half-siblings, and CKMR-based management procedures were provided. Also highlighted was that small-scale CKMR studies (e.g., studies that lack the statistical power to detect kinship pairs, given the assumed population sizes) are generally ineffective, and can be counterproductive unless sufficient effort is available to observe a few kinship pairs early on. Finding kinship pairs builds considerable confidence in moving forward. It was noted that missing years are not a major problem compared to the use of abundance indices, for example.

Discussion

A question arose of whether a formal study design was desirable prior to implementation of sampling. The responding comment was that such prior design is desirable, but that initial observations from CKMR can provide key insights into optimal design. Consequently, pilot sampling may be important to validate model assumptions and develop a successful study design.

3.2 WBFT CKMR

SCRS/P/2023/022 Streamlined approach for Close-Kin Mark-Recapture in Western Atlantic Bluefin Tuna

The workflow of genetics data processing for western Atlantic bluefin (WBFT) was presented (SCRS/P/2023/022). The keys to successful CKMR for WBFT, which followed protocols developed for southern bluefin, were identified as optimized biological sampling (including sub-sampling), extraction methods that included robotics, and well established and standardized database routines. The author highlighted the switch in DNA profiling that occurred in the process of developing the standard protocol for southern bluefin from microsatellites to single nucleotide polymorphisms (SNPs) using the Diversity Arrays Technology (DArT) approach, which had delivered the resolution capacity required for parent-offspring and sibling kinship relationships to eliminate false positives. Overall, CKMR of southern bluefin provided a template that has delivered ~3000 DArT-CAP DNA markers and methods now used for WBFT CKMR. The question was asked if the database format used for SBT and WBFT could be made available to provide a template for data collection on eastern Atlantic bluefin tuna (EBFT); it was agreed that this template would be shared. The high number of samples that would be necessary to apply CKMR in EBFT compared to WBFT, and the opportunity of having a single tissue bank were discussed. The presenter indicated that there is not a single tissue bank for WBFT, and that as long as a good common metadata base is maintained, there should be no difficulties.

SCRS/P/2023/008. West Atlantic Bluefin Tuna Close-Kin Mark-Recapture Abundance estimation

The results of pilot studies conducted for WBFT since 2015 were presented (SCRS/P/2023/008). The presentation addressed key uncertainties related to implementation of CKMR. The results of larval kinship, stock-of-origin identification, and parent-offspring pair analyses were presented across three years of sampling over 2016-18. The principle conclusions included the following: 1) larval collections provided effective marking events of active spawners from a known time/location; 2) catch stock assignment was achievable with relatively high precision; 3) analysis of larvae from the Slope Sea corroborated recent findings of mixed/hybrid breeding; 4) initial indications were that the CKMR estimate of West Area adult abundance is close to that from the current conventional stock assessment; and 5) the precision and accuracy of WBFT CKMR estimate will be improved with cross-cohort half-sibs (XHSPs) and additional cohorts of POPs and HSP data (2019-2022 pending). Overall, the research team indicated that operational implementation of CKMR for western bluefin is achievable in the very short term.

3.3 PBT CKMR

SCRS/P/2023/009. Close-Kin Mark Recapture (CKMR). An example of PBF

SCRS/P/2023/009 provided a summary of the ongoing CKMR application to Pacific bluefin tuna (PBF). Because this species is a single stock with multiple spawning grounds, the sampling for candidate offspring was conducted before the mixing of individuals from both spawning grounds, similar to larval sampling from the Gulf of Mexico (GOM). In addition, the authors introduced a way to include the CKMR data into the current stock assessment framework (Stock Synthesis) to investigate the performance of CKMR.

The Group highlighted the utility of integrating CKMR data into the assessment model framework, particularly to evaluate the information content compared to traditional data sources (e.g., CPUE, size compositions, age compositions) and the influence of the estimates of scale in relation to other data. It was also pointed out that analysis of the CKMR data external to the assessment is important to be able to compare absolute abundance estimates without the influence of fishery dependent data sources.

3.4 Other species

SCRS/P/2023/015 Close-Kin Mark-Recapture for spawning stock biomass estimation of Northeast Atlantic demersal species

SCRS/P/2023/015 presented the development of CKMR studies for Atlantic and Mediterranean fish species. CKMR is based on the principle that the larger a population, the smaller the probability that kin relationships will be found between individuals in a random sample of that population. This probability depends on species-specific characteristics that need to be considered, such as fecundity and mortality. Therefore, evaluating the utility of CKMR for a given species requires assessing existing knowledge on stock connectivity, availability of biological data, and accessibility to the number of samples needed to find sufficient kin relationships for the analysis. Specifically, in this study, the possibility of using CKMR in various commercially exploited fish species within the Atlantic Ocean, such as anchovy, sardine, horse mackerel, mackerel, megrim, hake, white anglerfish, yellowfin tuna and bigeye tuna, was evaluated. With that goal in mind, available biological knowledge was gathered for each species and used to calculate the number of samples required for each to be able to apply CKMR successfully. A further objective was to develop a CKMR study for two of the more likely species, hake and white anglerfish, and to estimate the magnitude by which some uncertainties in the input data might affect the results. The analyses showed that, although all investigated species are compatible with the fundamental requirements for CKMR, the required number of samples is logistically impossible for some of them. These results set the foundation for future CKMR studies aimed at supporting more informed assessment of fish stocks in the Atlantic.

4. Review of tagging studies to determine distribution and movement patterns

SCRS/2023/030. Revisiting hypotheses of population structure within the Mediterranean in the light of newly available electronic tagging studies

SCRS/2023/030 revisited hypotheses of population structure within the Mediterranean, which is important to inform CKMR study design. This study was based on the GBYP tagging database, which is a compilation of the international tagging effort and data on electronic tags. Specifically, using this extensive database, the authors investigated hypotheses related to the following: i) the usage of the Mediterranean by different size classes; ii) the fidelity to Mediterranean spawning sites; and iii) the difference in Atlantic migrations made by individuals from different areas of origin in the Mediterranean. Results showed that size affected the migratory behavior and

Atlantic/Mediterranean transitions, that fidelity to Mediterranean spawning grounds could not be rejected and that no differences in Atlantic migration could be detected between individuals from different likely areas of origin. The results also emphasized the need to continue electronic tag deployments in the Mediterranean, where key information is still missing at present, particularly in the Eastern region.

The Group thanked participants for their efforts to put the database and analysis together as this is very useful to help design the CKMR study. However, it was also mentioned that there are other sources of information (e.g., conventional tags, historical fisheries data, etc.) that are also informative to complete the picture provided by e-tags. A further comment was that migration dynamics are rather complex with several specific behaviours noted depending on location (e.g., an undefined number of bluefin tuna reside year-round in Gibraltar), but that the Group should aim to summarize general migration patterns.

There was some discussion on Mediterranean residency and the few data available from the Eastern Mediterranean, which have confirmed some migrations from this area to the Atlantic and vice versa. A comment made was that tagging after the spawning season would increase the chances of observing Mediterranean residents, as the migrants would already have left. However, this would not necessarily result in a representative sample of the population of spawners. Nonetheless, it is important to increase the times at liberty and the tags deployed on Eastern Mediterranean fish, to learn more about the resident behavior, specifically whether there could be some unconfirmed ontogenetic, sex or other life history differences between "resident" and migratory contingents (sensu Secor 2015).

The Group discussed some of the take home messages that could be important for CKMR. On the one hand, the fish from the western and central Mediterranean seem to have a comparable distribution in the Atlantic, so they may have a similar probability of being caught in the Atlantic. This might not be the case for sampling within the Mediterranean, as fish from a given spawning ground could be more accessible and likely to be caught around their respective spawning grounds (especially for western Mediterranean fish). Nevertheless, additional adult samples from Mediterranean spawning grounds could provide useful CKMR information on spatial structure and site fidelity. Unfortunately, the Group had very limited information from eastern Mediterranean fish.

It might also be that the size-dependent migration behaviour observed in the Mediterranean (where the largest fish perform the longest migrations) also occurs in the Atlantic. The Group was reminded that the document omitted migrations that did not enter the Mediterranean, but there are archival tags that suggest that some fish only enter into the Mediterranean later than the presumed maturity for Mediterranean fish. This would, however, be accounted for automatically in CKMR, because it would affect mean fecundity-at-age which, when multiple cohorts of potential offspring are sampled, is determined mostly by the retrospective detection of offspring of an adult in previous years. A CKMR model would normally exclude comparisons between an adult sampled on a spawning ground and any potential offspring born in that same year of sampling.

Overall, the Group agreed that there was no intrinsic spatial complexity in EABT to prevent proceeding with CKMR, providing that a few (but enough) key fisheries are able to provide enough samples, and that the method may elucidate many uncertain aspects of BFT biology that cannot be addressed with conventional sampling methods (e.g., reproductive success by size, or fidelity to spawning area).

E-tagging informs the spatial distributions of Atlantic Bluefin tuna stocks

The Stanford University electronic tagging program summarized 25 years of effort deploying archival and satellite tags on bluefin tuna. The West Atlantic movement data from US and Canadian collaborators and East Atlantic collaborations (Ireland, Norway, England) has now resulted in approximately 600 electronic tag tracks with long durations (greater than 180 days) for large fish (greater than 200 cm CFL) in the West Atlantic, East Atlantic and the Mediterranean Sea. These tags demonstrated annual movements mostly between 0.8 and 1.5 years-at-liberty for satellite tags, and some long archival tag tracks up to 5 years in length. These tag data, when aggregated together, demonstrate movements that can inform Atlantic bluefin tuna stock structure. When examining these electronic tag data sets some remarkable spatial patterns emerge.

The data demonstrated strong support for a Gulf of Mexico spawning aggregation from tags deployed primarily in Canadian waters on large fish. A Mediterranean spawning aggregation was identified that was composed of fish from Canada and North Carolina, tagged in the West Atlantic, and fish tagged in the East Atlantic offshore of Ireland, Norway and the UK. Electronic tagging data demonstrated that spawning of Eastern Atlantic fish occurred in the western and central Mediterranean. In addition, tagging data supported hypotheses of spawning in the Slope Sea by fish released from North Carolina and Canada that are of large mean size. Finally, seven long tag tracks from satellite tags deployed in the eastern Mediterranean were shown that all remained entirely in the Mediterranean; these were on fish of smaller mean size (approximately 143 cm CFL).

The Stanford team showed quarterly movements of the data from all tags, informed by inferring spatial spawning populations based on the spawning location in the tag track (GOM, MED, SS). The tags also recorded proxies for spawning behaviours in all regions where spawning is known to occur. The tag data can inform the CKMR process by identifying regions where proposed sampling could encounter multiple spawning populations compared to times and areas where single populations may be spatially segregated. These data show that it is feasible to use satellite tagging to obtain annual and multi-annual tracks with improved durations of attachment and high success through capacity building.

The Group noted that one individual assigned to the Slope Sea spawning location had also visited the Mediterranean during the spawning season, and recommended that the authors assess behavioral data (i.e., vertical immersion oscillatory patterns) to be able to better infer whether there were signs of spawning in both areas, or not.

The authors also highlighted some of the typical behavior patterns observed, such as fish remaining for several years in the West Atlantic before migrating to the Mediterranean to spawn, which are similar to observations of bluefin in the Pacific. Oceanic productivity hotspots are thought to be important factors in BFT distribution.

In relation to genetic studies (see section below), the authors were asked whether they had observed increased numbers of Mediterranean fish in the west over time. The authors mentioned that observing fish with migrations to the Gulf of Mexico is less frequent now compared to historical samples, while observations of fish that use the Slope Sea and Mediterranean are now more frequent. In the Western Atlantic locations where tagging was undertaken by the Stanford team, the fraction of the Gulf of Mexico population being tagged is decreasing and the fraction that is from the Mediterranean population has increased in the recent period. The Slope Sea behavioral pattern is also becoming more common.

Other participants emphasized the need to have a complete view with all e-tag data, as requested several times by the SCRS. There is also interest in observations of fish visiting west Africa. It was pointed out that some etags deployed within the GBYP program in the Eastern Mediterranean during the spawning season showed migrations to the western Mediterranean and the Atlantic Ocean.

The authors were also asked about when the tagged fish, for which fin clips are available, could be assigned genetically to stock of origin. The authors responded that they keep improving their genetic methods.

Some participants noted that the document, even in the form of presentation, had not been uploaded in the meeting own cloud folder, and requested the authors to make it available to the Group.

5. Review of genetic studies on population connectivity

SCRS/P/2023/016 Population connectivity of Atlantic Bluefin Tuna

Early genetic information-based population connectivity analyses supported the hypothesis that ABFT exhibit natal homing behavior and allowed for the development of a 96 SNP based stock identification tool (Rodriguez-Ezpeleta *et al*., 2019). Additional analyses based on more samples, including larvae from the Slope Sea, revealed the existence of mixing between individuals from the two main spawning grounds, resulting in individuals with admixed genetic profiles, mostly concentrated in the Slope Sea. It is still not clear how genetic differentiation is maintained in the presence of gene flow. Two hypotheses have been advanced: 1) a recent expansion (either mediated by an increase in population size or by an improvement of conditions in the Slope Sea for reproduction) of the Mediterranean population into the West, after which there has not been enough time for homogenization; and 2) a selection against admixed individuals. These hypotheses are currently being tested through additional analyses, notably those based on Whole Genome Sequencing of 25 bluefin and 2 albacore tuna individuals. One conclusion from these findings is that, in the long term, continued monitoring of population connectivity is needed to determine the extent to which the western population is dependent on the eastern (i.e., is there evidence of source-sink dynamics). In the short term, the Group needs to be aware that stock identification methodologies are imperfect, and that they continue to improve as our understanding of the stock increases and as genetic tools become more powerful.

Discussion centred around the differences between evolutionary time frames and those relevant for the assessment. Specifically, the timing of the expansion of the Mediterranean population into the West was discussed in the context of increased presence of eastern fish detected in the West Atlantic using different methods, including satellite tagging, genetics and otolith microchemistry during the recent decade. The genetic data support the hypothesis that Mediterranean and Gulf of Mexico genetic components have been isolated in the long term, but the duration in years for a hypothesized genetic mixing between the stocks cannot be estimated.

6. Reproduction/fecundity at age and identification of spawning sites as it pertains to CKMR parameterization

SCRS/P/2023/014. Brief synopsis of eastern Atlantic Bluefin tuna reproductive dynamics

An overview of bluefin tuna reproductive parameters, sex ratio and age at maturity of the eastern stock was presented. The paper included the different approaches available (direct and indirect) to estimate these parameters. A summary of the most reliable values of these parameters was presented; these are detailed below.

- Age 50% maturity = 3-4 years; Age 95+% maturity > 4 years
- Relative batch fecundity ≈50 eggs g^{-1} body weight (this ratio is likely to be consistent over SFL range 95-220 cm)
- Estimated duration of the spawning period ≈ 24 days
- Spawning frequency (days between consecutive spawning events) ≈ 1.2
- Seasonal number of spawning events ≈ 20
- 1 kg of fish → 1 million eggs; A 100-kg female ABFT may potentially spawn ≈100 million eggs over the spawning season.
- Sex ratios. 90-170 cm SFL: SR ≈1:1 (F≈M); 170-220 cm SFL: F>M; >220 cm SFL: F<M

The estimates of the spawning duration using different approaches differ, but it was pointed out that considerable variation was observed between sizes and regions. It was also pointed out that the contribution by age and sex can be estimated by CKMR.

The presentation also covered other topics, such as the challenges associated with the possibly uneven distribution of the spawning population into subunits showing different individual sizes and migratory patterns. Some particular features were enumerated, including the origin of small juveniles caught in the Canary Islands, the larvae found in the Gulf of Biscay, and the spatial-temporal structure of ABFT around the Straits of Gibraltar.

The Group concluded that the hypothesis that young eastern ABFT do not undertake long reproductive migrations to principal spawning grounds, but instead spawn outside the Mediterranean Sea, cannot be rejected. Pacific bluefin have similarly shown segregation of spawners by age/size in three different breeding grounds of the Sea of Japan. During the discussion, the Group was reminded that the tuna trap in La Linea (southern Spain, close to Gibraltar) usually catches juvenile bluefin tuna migrating out of the Mediterranean, with those tuna possibly being found later in the Bay of Biscay.

7. Synthesis of current knowledge on eastern and Mediterranean stock structure (discussion)

The Group reviewed information provided through electronic tagging, genetics and reproduction studies (see sections above). Studying subpopulation structure within the Mediterranean is challenging because, among other reasons, no genetic differences are observed between potential subpopulations (individuals from different spawning grounds). However, recognizing all the uncertainties, the Group agreed on the following working hypotheses to use for the application of CKMR:

- There are different spawning areas throughout the Mediterranean, including the Balearics, southern Tyrrhenian, south of Malta and the Levantine Sea, with additional opportunistic spawning grounds.
- There might be some fidelity to spawning areas. However, fish from the Western and Central Mediterranean use similar areas in the Atlantic and thus might have a similar probability of being caught while in the Atlantic (in other words; Atlantic sampling sites might be well-mixed).
- Within the Mediterranean, there is a chance that the probability of capture is greater around the spawning area of origin. There is little information about the Eastern Mediterranean, and it is unclear whether fish from that area have different spawning dynamics and migration patterns, possibly induced by the different oceanographic conditions.
- Mention was made that migration from the Mediterranean to the Atlantic and vice versa is possibly exhibited mainly by fish larger than around 175 cm, but old trap data does not support this hypothesis.

8. Review of genetic methodologies for stock ID, kinship analyses and sex determination.

SCRS/P/2023/026 Atlantic Bluefin stock structure and Kinship among three larval year classes sampled from Gulf of Mexico and Balearic Islands

SCRS/P/2023/026 describes the evidence for stock structure among three larval years classes from the Mediterranean and the western Atlantic. The researchers were able to obtain good quality DNA from larvae collected during the standardized Balearic survey for use in population structure analysis. One hundred loci were developed for stock ID using the DaRT-CAP approach. The data suggest that there are clear differences in the genetic signatures for the Mediterranean and the Gulf of Mexico larvae, and that larvae from the Slope Sea appear to be a mix of exclusively western, exclusively eastern and possibly hybrid east-west origin. Three sampling locations for use in the western CKMR project have been identified, and some kinship pairs have already been found within and between cohorts from these locations.

In the discussion after the presentation, the desirability of a future meeting to address the different genetic techniques that are being used on both sides of the Atlantic for ABFT was mentioned.

SCRS/P/2023/018 Methodologies for ABFT Stock ID, kinship, sex determination and others

Two genotyping tools have been designed, validated and applied to monitor Atlantic bluefin tuna under the GBYP program during phases 5 through 12. The first tool consists of a 96 SNP chip designed to assign natal origin of Atlantic bluefin tuna to either the Gulf of Mexico or Mediterranean Sea spawning areas, based on the assumption of two genetically isolated stocks. The tool was validated using individuals that were not used for marker selection. An improved baseline was generated incorporating more larvae from the Gulf of Mexico, which resulted in a similar proportion of unassigned individuals. Taking into account the results obtained from the population connectivity studies (see section 5), which probed admixture in the Slope Sea and the presence of pure Mediterranean genetic profile individuals in the Gulf of Mexico, the baseline was rebuilt based on individual genetic profiles obtained using thousands of SNPS. Furthermore, a new subset of SNPs for stock identification was selected based on these new criteria, and three markers for genetic sex identification adapted from those developed for PBFT (Suda *et al.*, 2019; Chiba *et al*., 2021) were included. This selection was validated based on samples of known origin that had not been used for marker selection, and was applied to assign genetic origin of >3700 individuals from feeding aggregations. Markers for sex identification were tested on 50 samples for which sex was confirmed by gonad histological inspection, resulting in 97% sex assignment success.

The second tool consisted of a SNP array including 8,000 SNP markers, which was designed and validated to obtain population-structure-relevant information (such as complete genetic profile and potentially adaptive markers), kin finding, sex determination and mitochondrial variant analysis. More than 700 individuals had been genotyped using this tool, including individuals from the Mediterranean Sea, the Gulf of Mexico and feeding aggregations in the Atlantic. Kinship analysis performed on these individuals revealed the suitability of this tool for kin detection. Several half and full sibling pairs were detected among the samples analyzed.

The main point discussed was the fact that some of the research centers involved in ABFT genetic studies are currently using different genotyping approaches for finding kin; however, replication across different genotyping platforms had corroborated the alternative approaches and increased confidence in both stock-of-origin assignment and kin-finding applications to ABFT. The west is using a genotyping approach for Stock ID and kinship analyses that is based on approximately 3500oci genotyped through DArTcap™. This is similar to the DArTseq™ method, where a reduced representation of the genome is sequenced, and probes are used to "capture" the loci (i.e., markers) of interest, with only those being sequenced. The raw sequences obtained are then bioinformatically analyzed and the SNPs are analyzed (genotyped) for each individual and loci. The east is using a genotyping approach for Stock ID and kinship analyses (and other applications such as sex determination, albacore introgression detection) that is based on approximately 8000 SNPs (~7400 valid for kinship analysis), genotyped using probes synthesized in a microarray substrate.

Using different loci and methods implies that the samples analyzed by the western project and those analyzed by the eastern project cannot be directly compared for kinship unless some samples are re-genotyped using the other method. It was clarified that the loci developed for the western project are valid to find kinship in the east and vice versa; this is not a matter of which loci work better, since both sets appear to work well for eastern and western stocks.

The possibility of having a common approach for East and West was raised. This would allow for the combination of datasets and finding kin pairs between samples analyzed by each of the projects. This could be particularly useful, for example, for Canadian samples, which are highly mixed and contain individuals from western and eastern stocks. Such comparisons are likely to become critical for testing the hypotheses posed above regarding potential genetic mixing of the Eastern and Western (or other) populations, and would have value in increasing the effective sample size for both East and West. A unified approach would be beneficial from an efficiency standpoint. One possible way to do this would be to include the western loci in the eastern array. This is possible in theory and could represent a path towards a unified pan-Atlantic genomic assessment of ABFT. However, it may not solve the issue in practice, because despite using the same loci, the technology would be appreciably different (one probe-based and the other sequencing-based), which could result in slight differences that could affect kinship findings. The Group noted that several options remain: 1) maintain separate projects; 2) maintain separate processes but share samples, providing replication and method comparison and validation; 3) integrate the Western markers into the Eastern process; and 4) make a new single CKMR project for the species. **Appendix 3** outlines these options further; the Group agreed that the options should be evaluated further in light of costs and benefits, as well as both short-term and long-term directions of the science. The interest of several additional research laboratories in Japan, Germany, Italy, UK and USA to cooperate in the CKMR analyses was also noted.

9. Review of the potential of epigenetic aging for ABFT

The invited expert advised that CKMR requires age (and/or in some cases body-size or life-stage) information for each sample. While these age measurements do not necessarily have to be exact (i.e., accurate to the spawning season), more accuracy generally leads to more precise and reliable estimates of demographic parameters in CKMR. Accurate age is most important for potential offspring, and somewhat less so for large/old adults. The worst problems that the expert had encountered with CKMR originated from highly inaccurate age and/or length data. In the case of EABT, larval age is known, and for young juveniles (say, 2–4 years old) length alone might be adequate (as with SBT, for example), but for the adults (i.e., potential parents) more accurate age information will be important. It is not always logistically possible to collect otoliths, which are also somewhat costly to read; thus, alternative methods such as epigenetic ageing are potentially attractive.

The Workshop heard two presentations on this topic: a review (desk study), plus recent results for SBT. The discussion of both is presented at the end of this section.

9.1 Desk study

Assessment of the potential of epigenetic approaches for ageing Atlantic Bluefin tuna samples

Previous work has suggested that methylation profiles could be used to assign age in commercial fish species. Yet the age estimates obtained have sometimes had quite high errors (when compared to otolith derived ages), which limits the utility of this method for the Atlantic bluefin tuna assessment, including for future CKMR endeavors. Here, only the potential of epigenetic approaches for ageing ABFT individuals is evaluated. Accordingly, a bibliographic search was performed to compile all studies using epigenetics for aging fish. The information obtained has been contrasted with the data required for assessment and CKMR to evaluate the potential of this approach, and to identify its advantages and disadvantages over existing methods.

There was a brief discussion on the ageing method for albacore and swordfish used to calibrate the epigenetic ageing developed by IFREMER. The suggestion was made that spine ages may be more accurate than otoliths for ageing swordfish.

It was noted that a publication will appear soon on the targeted bisulfite sequencing approach for epigenetic ageing, and those methods and results can then be shared.

There was some discussion on the potential cost of the epigenetic ageing assay. It was noted that a cost of 10-20 euros per sample may be possible, after the initial investment to develop the method. More precise age estimates could be obtained by investing more funding into sequencing more regions of the genome. It was noted that the SCRS needs to consider the cost of epigenetic ageing compared to otolith ageing, which can be expensive when the cost of extracting, preparing and reading the otoliths is considered.

Assuming the same tissue sample is used for CKMR and epigenetic ageing, the cost of sampling and DNA extraction would be shared across methods, reducing the cost of epigenetic ageing, and hence contributing to making it more competitive from the financial point of view compared to otolith ageing.

It was noted that small fish may not need to be aged as length may be good predictor of age; but for larger fish, direct ageing (at least of a substantial subset) is required, either via otoliths or tissue samples. In general, better precision in age estimation will translate into smaller overall sample size requirements, but the trade-offs are subtle. The various cost-benefit trade-offs can be investigated during the design phase of a CKMR project.

9.2 Previous studies and presentation of an ongoing study

SCRS/P/2023/021 Epigenetic Ageing in Fish

SCRS/P/2023/021 focused on the general methodology for epigenetic aging which relies on changes in the methylation state of various loci over the course of an organism's life. CSIRO is developing a cost-effective rapid PCR assay, targeting informative sites (as identified through comparisons with other ageing methods). In some cases, it is possible to use sites identified in other fish when developing an approach for a new target species. The efficacy of this method depends on the evolutionary relationship between the species and the number of conserved sites.

It was noted that epigenetic ageing has been successfully performed for southern bluefin, yellowfin and bigeye tuna, though work has not yet started for Atlantic bluefin tuna. The same markers were used for all three tuna species studied, and it is very likely that these markers will also work for Atlantic bluefin tuna. The markers used are highly conserved between tuna species.

Confirmation was provided that ABFT eggs and larvae might be useful for epigenetic ageing (age 0 fish); although it was noted that there is less DNA in non-somatic tissue. Nevertheless, it was pointed out that eggs developed rapidly to the embryo stage, thus providing more than ample amounts of DNA for this purpose, and that eggs have been successfully used in microsatellite determinations.

The method currently being developed by CSIRO is restricted to a single tissue type (e.g., muscle tissue). Future work could be explored using multiple tissue types, but developing the method would be more expensive and require more probes, and thus cost more.

There was some discussion on the need for uniform distribution of age classes in the training data (comprising perhaps 100-200 fish total). The presenter commented that the method is based on machine learning, so if there is a gap in the age distribution, then the age prediction involved might not perform as well for the missing age classes. However, for SBT there are no gaps and there should be no problem in obtaining a good spread of ages in a training set for ABT.

For ABT, it might be possible to obtain known age fish from the farming pens. These samples would be very useful in age calibration work, particularly if the same fish could be sampled over multiple seasons or years. In addition, tissue samples from fish that have been tagged and recaptured (samples from two time periods from fish of known time at liberty) would help to assess prediction performance. It was confirmed that samples from wild caught juveniles that had been kept in captivity for many years might be available. It would also be possible to sample these fish over time to determine in addition how well the prediction was performing. There are methods now available for sampling captive fish over periods of time underwater, and the provision of brood stocks for aquaculture will enhance this prospect.

It was noted that it may be possible to test if tissue samples from different parts of the fish (e.g., the head versus the tail) affect age prediction. It was noted that the differences are likely to be low compared to other samples such as those from the internal organs of the fish.

Confirmation was provided that a sex ratio of ~1:1 was desirable for the age calibration work in case there are sex-specific differences in the process. It may be possible to determine the sex of fish genetically to ensure the correct ratio in the analysis.

Information was offered that the updated epigenetic age data presented for southern bluefin tuna using a new statistical approach developed by CSIRO was extremely good. It was noted that the otolith-based age estimates may be relatively accurate (or have low error) since the graph of epigenetic age versus otolith age looks so good. It was also noted that since the epigenetic ageing shows promise, the magnitude of the errors observed might not require any improvement and might be entirely sufficient for CKMR purposes.

The presenter commented that the preliminary results for yellowfin tuna appeared to be just as impressive as for southern bluefin tuna. Transferring the method has worked well between these two tuna species, and it is expected to be just as good for Atlantic bluefin tuna. The presenter confirmed that the transfer process is not very expensive and that there has been no need to search de novo for additional loci.

There was some discussion on the number of markers used for the tuna species. It was noted that the whole genome approach will find more sites. However, the presenter confirmed that of the 1300 CpG sites previously found in zebra fish that correlate with age, only 100-200 are retained across species.

There was discussion of the error rate for epigenetic ageing relative to an age length key (ALK). There is currently no ALK used in the stock assessments for Atlantic bluefin tuna, and cohort slicing is used to age fish (annual ALKs have been generated since 2010, although the years and size range is inadequately covered, especially in the eastern Atlantic ALKs). Cohort slicing has a very high error rate, particularly for large fish. It was noted that epigenetic ageing would be a major improvement, even if there was some error. The relatively high cost of otolith ageing was noted, as well as the difficulty and cost of collecting otoliths in the numbers required for ABFT. Annual ALKs require large numbers of age-at-length estimates. Epigenetic ageing may be a game changer for implementation of CKMR. It was noted that there is a need for another workshop in the future to discuss genetic methods for CKMR, including epigenetic ageing.

Confirmation was provided that the GBYP pilot study on epigenetic ageing techniques for ABFT would focus on the full age range of fish, for both main stocks and for both sexes. The quality of tissue samples was noted to be very important. Approximately 250 samples per stock (500 in total) are planned to be analyzed.

10. Biological sampling

The EBFT chair explained that initially this workshop was envisioned to include both a discussion of CKMR and a general discussion about biological sample collection. Some participants noted the absence of people from the different CPCs to be able to address the discussion of a general sampling program, as well as the limited time available at the workshop; this led to the suggestion to continue discussing general sampling at a future date.

To assist in the sampling design and to see the possibilities of sampling by fleet, the fisheries data department of the Secretariat prepared figures showing the spatial distribution of catches by gear in the Mediterranean from 2010 to the present. It also presented the size distribution by the Mediterranean fleet/gear groups, based on the input data from the Stock Synthesis model applied in the EBFT assessment conducted in 2022 (Sampedro *et al.*, 2022).

10.1 Description of current biological sampling

10.1.1 ICCAT GBYP existing programs. Tissue Bank.

SCRS/P/2023/017 GBYP biological sampling

SCRS/P/2023/017 described the current GBYP biological sampling program which has been in place for the last 12 years. GBYP samples are maintained in a tissue bank at AZTI where they take charge of sample coordination, sample reception and sample storage (including keeping replicates in separate locations). Information on this collection is available at https://aztidata.es/bluefin. This presentation also described the EU Data Collection Framework for fisheries implemented by EU DG-Mare (DCR and DCF). The EU has created a framework for fisheries data collection in which data from member states are made available to support the Common Fisheries Policy and RFMOs. DG-Mare is currently working on an updated IT platform for these data. The presentation also provided information on the DCF funding of larval surveys in the waters around the Balearic Islands.

The Group noted that the detailed location of the biological samples collected under the European framework is still unknown and that it would be useful to get this information. It is unknown how the current sampling programs would support a CKMR study, but it was noted that it would be better to first design the sampling for the CKMR study and then ask for its implementation. The Group highlighted that the sampling should be undertaken by the CPCs with coordination by the GBYP, but that due to cuts in the GBYP Program, the GBYP would not be able to take over the payment for the sampling. Regarding the use of the catalog of samples available through GBYP and DCF for the application of CKMR, clarification was provided that recent larval and juvenile samples could be used, but that adult samples collected previously would not be useful if not duly collected for this purpose or if sample sizes were small.

10.1.2 CPC sampling programs. EU data collection framework (DCF), others.

EU-DCF biological sampling

Addressed in presentation SCRS/P/2023/017, as described above.

SCRS/P/2023/027 Current larval sampling in the Balearic Sea and larvae suitable for DNA extraction

SCRS/P/2023/027 presented the current larval sampling strategy employed in the Balearic Sea. In a normal year, the salinity front and where it aggregates the larvae around the Balearic Islands will be identified. Samples are then taken from these specific areas based on the habitat modelling. Currently, half a tow (i.e., the sample from one bongo net) is preserved in formalin and the other half in ethanol. The survey can collect thousands of larvae in a single tow, and there is the possibility of collecting more larvae at a given station through additional tows if that was required.

There was some discussion about whether it would be beneficial to increase the sampling effort, but it was determined that this was probably not necessary at this point). It was further discussed whether ensuring sampling in different areas of the Balearic Sea was important for obtaining the larval offspring of different spawners. It was pointed out that it is important to obtain high quality preserved samples representative of as many unique spawners as possible, so subsampling at all stations is better than taking all the samples from a single tow. The Group also recommended taking large larvae that have more tissue for genetic analysis, and which have also spent greater time in the environment for mixing between the spawning and sampling events. The Group noted that it would be useful to extend the larval sampling to additional Mediterranean areas as detailed in the point that follows.

10.1.3 Type and place of storage of the biological samples available (EU-DCF Data call).

Since the DCF biological sampling results are not collected in a common database/tissue bank, the Group proposed to make a request, via Data Call to DG-Mare, to be advised on what kind of biological samples are available, where they are stored, and in what state of preparation and processing.

10.2 Future sampling planning

10.2.1 Design based (e.g., Systematic grid, etc.) or targeted sampling

The fundamental assumption of CKMR is that one of the sampling events is a 'random sample' with respect to the population of spawners. This can either be the adult or the juvenile (e.g., larval) samples. As shown in McDowell *et al.* (2022), larval sampling is not random relative to the distribution of parentage as there is a moderate degree of within and between tows relatedness. Ideally larval sampling would follow the design-based approach of the survey, allowing samples to be used both for CKMR and for the standardized larval indices currently used in the stock assessment and the adopted management procedure. However, given that the larval samples are not random, and given the relatively high number of spawners per event observed, it is possible to augment numbers with adaptive sampling in areas of high larval abundance. McDowell *et al.* (2022) tested both approaches and found that the design-based approach should be preferred and had the lowest sibship, but that adaptive and even targeted samples could be used to increase the total number of samples for CKMR. While mathematically more complicated to account for the error structure of correlated samples (McDowell e*t al*., 2022), the ability to target very large numbers of larvae may outweigh the loss of information due to relatedness.

10.2.2 Types of biological samples (calcified structures, gonads, genetic samples, etc.). Biological sampling Data Base - Tissue bank.

For CKMR, only genetic samples are required together with the associated metadata on length, location, gear and date. Ideally this should be paired with a calcified structure, to compare age estimation and visual sex identification of gonads for gender confirmation. If genetic methods are able to provide an estimate of age (through epigenetic aging) and gender (through sex ID markers) and these values are validated, in the future all that is needed will be excellent quality genetic samples.

10.2.3 Opportunities for sampling in large numbers. Larvae, juveniles, adults.

SCRS/P/2023/023 Larval sampling opportunities

Presentation SCRS/P/2023/023 summarized the results of the meeting of the larval ecology groups to assess the possibility of providing samples for the CKMR. Research groups working on early life ecology of tunas in the ICCAT area participated in a meeting in March 2023 organized by GBYP to review all the ongoing activities, identify common research objectives, and advance towards standardized sampling and data analyses protocols. Six bluefin tuna spawning areas have scientific groups conducting research activities. From east to west these areas are as follows: 1) in the Mediterranean: South of Turkey, South of Sicily, Tunisian waters and the Balearic Sea, and 2) in the Western Atlantic: the slope Sea and the Gulf of Mexico. Sampling activities in the Gulf of Mexico and the Balearic Sea are supported by dedicated data collection frameworks for tuna species; in the other areas the sampling is conducted under annually funded initiatives or In collaboration with other sampling programs. All the research teams indicated willingness to contribute to the collection of samples for the CKMR study. The total number of larvae collected in the different areas per campaign is very different (from 50 to >20.000). The research teams are working towards increasing catches in all areas by standardizing effective fishing protocols and applying adaptive sampling designs targeting specific oceanographic features. Improving the coordination of sampling activities among the larval ecology groups will have a positive impact on CKMR, and to this end these independent research groups have jointly suggested that a larval subgroup be created within the SCRS BFT Working Group.

It was noted that the Balearic Sea samples constitute adequate numbers of larvae to provide high numbers to CKMR. However, to have samples that are representative of the entire Eastern bluefin tuna stock, it would be desirable to include samples from other areas, especially from the Eastern Mediterranean (programs for the South of Turkey).

The larval ecology meeting attendants requested guidelines which would help to design the sampling campaigns in order to benefit CKMR. The Group suggested that, for the purposes of characterizing the genetic diversity and potential spawning site fidelity, there is a need to obtain moderate numbers of larvae (e.g., 100s) from several spawning areas. However, for the purposes of CKMR, it will be imperative to collect high numbers (e.g., 1000s) of larvae, which may be possible for a few locations only.

The larval ecology group requested that the CKMR Group include a recommendation or statement declaring their high interest for maintaining and reinforcing the larval sampling activities on the different spawning grounds, similar to that expressed after the first GBYP workshop on larval surveys held in 2016. This declaration is of importance as it would help them to obtain funding from relevant national agencies.

SCRS/P/2023/020 ROP sampling possibilities. Mr. Thomas Franklin.

Presentation SCRS_P_2023_020 reported on opportunistic genetic sampling by compliance observers under the ICCAT Regional Observer Program. The presenter informed the Group that these observers could collect samples, when possible, if they were provided with adequate equipment and sampling protocols.

The Group acknowledged this possibility, but as mentioned before considered that sampling for CKMR should not be an opportunistic activity and has to be the primary goal of a dedicated scientific observer in order to guarantee not only the quality of the sample, but also the systematic collection of the required samples.

11. Sampling requirements for CKMR

For this section, the invited expert, Dr. Mark Bravington, was asked to provide some sampling scenarios that could be used to start the discussion around sampling requirements. He began with a reminder that it is not necessary to sample everywhere in order to implement CKMR, but that it is necessary to sample from enough places to allow for the testing of spatial structure. The working hypotheses that were presented had an implicit assumption that there is some degree of faithfulness to the three main Mediterranean spawning areas (some evidence for this was provided in previous sections); and also that samples from the Atlantic would be well-mixed with respect to the different Mediterranean spawning areas, at least for the largest /oldest fish. Six potential sample sites were proposed as follows.

Location	*Type*	*Mixing*	*Sample Size*	*Limitation/benefits*
Balearic larvae	Juvenile	Pure	Large	Moderate level of intra-cohort siblings
Croatian 2-4 years	Juvenile	Impure CMed (+West+East)?	Large?	Would extend juvenile cohorts back several years
Portuguese traps	Adults	Well-mixed	Medium	
Canada	Adults	Well-mixed	Small/medium?	~50% will be of Med origin
Norway	Adults	Well-mixed	Small?	Samples of large fish
Central Med (Malta)	Adults	Pure	Medium/large?	Possibly a limited size range
Balearic (Spain)	Adults	Pure	Medium?	Possibly a limited size range

Using samples taken from these areas (or their equivalents), it would be possible to obtain a Mediterranean-wide adult abundance estimate (conditional on the well-mixed assumption); this would also allow for a variety of hypotheses including well-mixing to be tested, and would provide information on the following parameters of interest.

- Determine if the use of spawning sites is maintained over generations (Heritability) – examine young adults [possibly using Croatian samples] to see if they are offspring of older adults. If spawning areas are heritable then there will be a considerable number of POPs would be found amongst central Mediterranean adults and amongst Balearic adults, but not many between areas.
- Determine if adults return to the same spawning grounds over subsequent years (Faithfulness) - if adults return to same spawning ground with high probability, then it would be not expected to see many adults from one site that are parents of juveniles from another.
- Test how well mixed the adult samples from different Atlantic sites are.
- Estimate overall abundance– key here is to have well mixed adult samples (i.e., use Norwegian / Canadian / Portuguese samples) – and compare any juvenile samples to these adults. This should result in the same rate of finding POPs regardless of the juvenile sample source. Similar results should be found between Norway/Canada/Portuguese adults.
- Estimate spawning-site-specific abundance if there is high faithfulness – compare adults and juveniles from the same site (i.e., Balearic juveniles to Balearic-caught adults, and Croatian juveniles to Croatian-caught adults). [caveat: this will not get much information about fish from the eastern Mediterranean]
- Estimate fecundity-at-size – this needs the full-size range of adults in sample; it might take a few years to catch enough adults that are sufficiently old to be a parent of juveniles that have already been sampled.

The presenter also suggested that it is best to use a full CKMR model, rather than trying to address aspects of interest individually. These aspects can be pulled out of the full model results. He also pointed out the importance of early discussions with the people who do the sampling. The following basic strategy was suggested:

- Go through a rigorous quantitative design process, including determination of logistic feasibility for the areas above and/or their equivalents;
- Without pre-empting results from the latter, there are some areas which are obviously key to any conceivable program (Balearic larvae being the most obvious example), so for those areas it makes sense to start sampling as early as possible [if this can be done through regular programs] and store samples for later genotyping [once the full project is approved and funds are available];
- It is important to sample across the adult size range, but an overall shortage of adult samples can be compensated by collecting many more juveniles – this is something to consider during the design process [exception: large adults are disproportionately valuable because they have been breeding for many more years and are expected to generate more kin pairs].

During the discussion, several other potential sources of samples were identified, including young-of-the-year (YOY) and 2-4 year old juveniles from the Croatian fishery, other YOY samples from the coastal recreational fishery, adult samples from the western Atlantic US and Canadian fisheries (of which substantial proportion are Mediterranean spawners), and juveniles from the French fishery in the Mediterranean. Egg samples are available from Malta for a number of previous years. These options can be considered. It was however noted that it is more urgent (and more efficient) to begin with juvenile sampling, since adults caught now will not be potential parents of future juvenile samples, but adults caught further into the future could be the parents of current juvenile samples.

There was some discussion about the merits of including more samples from other areas, but the expert pointed out that it is not necessary to sample everywhere and that it is on the whole better to have fewer sampling sites with large sample sizes of known origin, rather than many sites with smaller sample sizes, but each subject to an a priori unknown degree of mixing. For the adult samples of ABFT, the most important consideration is having at least one well-mixed location; this cannot be completely guaranteed in advance, but over time it would become possible to detect a major failure of that assumption from the CKMR data themselves. A decision not to use certain samples (at least in the beginning of a project) for reasons of efficiency and/or modelling simplicity, e.g., if not including samples from the western Atlantic, does not lead to a bias in the results. Because sample collection and genotyping does incur appreciable cost, and because adding too many sites with small sample sizes (especially for juveniles) could lead to overparameterization and an inability to test hypotheses, it is important to concentrate on sites where sampling is logistically efficient, where sample sizes are reasonably large, and where the "per sample statistical information" is reasonably high. It was noted again, by several participants, that a single Atlantic-Mediterranean-wide CKMR study should cover all different components of the various stocks, optimizing sampling efforts, analyses and costs.

The question of how long it would take to see initial results was raised. Starting the project is subject to approval by the SCRS and the Commission. Following approval, five years was suggested as a possible timeline, with the caveat that speed will depend in part on funding, and that use of juvenile samples already being collected (Balearic larvae) could also provide more cohorts for analysis at the start of the project. Provisional results might be available sooner, although within a shorter timeframe there would probably not be enough data to allow all assumptions (e.g., well-mixing) to be tested. These issues can, to some extent, be investigated during a one-year design phase implemented to cost out the project properly. It would be useful to think about how the CKMR timeline will fit in with the ICCAT management procedure review timeline as use of CKMR to inform recondition of the MSE would be a major success for the project. Information on the ICCAT BFT MP timeline can be found at ICCAT rec 22-09 https://www.iccat.int/Documents/Recs/compendiopdf-e/2022-09-e.pdf.

It was noted that formal design work after this meeting will be needed to specify actual numbers for the sampling requirements.

12. Recommendations about further steps

Vision: By 2027, provide initial CKMR estimates of pan-Atlantic absolute population size for ABFT (West and East) to inform potential reconditioning of the MSE operating models, address the greatest source of uncertainty in the MSE, and support the convention objective of maintaining biomass at the level that supports MSY.

Caveats: This vision and plan have not yet been considered by the BFT WG or SCRS, and achievement is contingent upon approval of the SCRS and obtaining the funding support necessary.

Aspirational Timeline

2023: Elaborate design plan TORs, consider bringing in external expert to GBYP SC
2024: Elaborate design, draft TORs, present to SCRS for approval and Commission for approval and funding
2025: Initiate EBFT CKMR, pending funding and design
2025-2026: Field seasons for sampling larvae/YOY and adults
2027: Deliver CKMR estimate to BFT WG

2023 Ongoing tasks

Tasks listed in 1-6 below were all outlined in the 2021 CKMR workshop report https://www.iccat.int/Documents/CVSP/CV078_2021/n_3/CV078030198.pdf], on which the SCRS, GBYP and national scientists have made good progress. Notes in bold identify progress or the party responsible.

1. Perform an epigenetic ageing study for BFT (using a reference collection) in order to check if this method works for BFT. If it proves not to work, otoliths will be needed, which would substantially increase the cost of sampling; it is even unsure whether it would even be possible to collect a sufficient number of otoliths.

2. Genotype at least 1000 Balearic larvae to determine kinship within and between tows. Use this preliminary information to evaluate an optimal design.

3. Identify suitable candidate fisheries (high yield) and field test protocols for CKMR. The project should not commence before confirming realistic and feasible sampling options. Sampling protocols also need to clearly identify methods to avoid DNA cross-contamination and ensure high quality samples. A sampling protocol for CKMR with technical details should be written.

4. Increase current (2023) sampling efforts focusing on larvae and juveniles (identify high yield, highly mixed areas and established sampling programs), ensuring that new larval samples will follow protocols that allow they can be used for CKMR (see 2023 larval workshop report.]

5. Archiving of larvae and adult samples in a GBYP tissue bank until funds can be obtained to genotype.

6. Explore the feasibility of obtaining additional larval collections in other spawning areas.

***NEW* 2023 tasks**

7. Elaborate TORs for a model-based sampling design for EBFT CKMR [GBYP].

8. Appoint an external expert on genetic methods and CKMR to the GBYP SC to assist in decision making [GBYP].

9. Conduct side-by-side comparison of existing genetic methods on paired fish to evaluate: a) detection of cross-contamination, b) stock differentiation ability, c) kinship detection, d) the possibility and consequences of merging or not merging methods. (**Appendix 3**) and e) provide some cost estimates to inform a call for tenders, which should ensure a broad participation of laboratories.

10. Evaluate whether the existing samples and data can be used to evaluate hypotheses related to CKMR spatial structure. [GBYP consortium-AZTI].

11. Identify funding opportunities for the proposal of CKMR for EBFT.

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Appendix 1

Workshop on bluefin tuna close-kin mark-recapture (CKMR), including biological sampling coordination
(Madrid, Spain, hybrid, 14-16 March 2023)

Agenda

(Coffee break 10:30 am, lunch 1-2:30 pm, coffee break 16 pm)

1. Opening, adoption of agenda and meeting arrangements

2. Brief overview of CKMR [rapp- GBYP, C. Fernandez]

   – Close-Kin Mark-Recapture and Eastern Atlantic Bluefin Tuna. Mark Bravington (Day1)

3. Relevant case studies [rapp- GBYP]

   a. SBT CKMR
      – Key considerations and lessons learnt from the implementation of CKMR for SBT: 2006-2023. Campbell Davies (around 9-11 am CET , Day 1)

   b. WBFT CKMR
      – A streamlined approach for the CKMR approach established for Western Atlantic Bluefin Tuna, based on 5+ year classes of adult and larval sampling. P. Grewe (around 9-11 am CET , Day 1)
      – SCRS/P/2023/008. West Atlantic Bluefin Tuna Close-Kin Mark-Recapture Abundance estimation. M. Lauretta (around 9-11 am CET , Day 1)

   c. PBT CKMR
      – SCRS/P/2023/009. Closekin Mark Recapture (CKMR) An example of PBF. Yohei Tsukahara (Day 1)

   d. Others, time permitting
      – SCRS/P/2023/015 Close-Kin Mark-Recapture for spawning stock biomass estimation of North-east Atlantic demersal species Iker Pereda

4. Review of tagging studies to determine distribution and movement patterns [rapp- GBYP, ???]

   – SCRS/2023/030. Revisiting Hypotheses Of Population Structure Within The Mediterranean In The Light Of Newly Available Electronic Tagging Studies. Tristan Rouyer.
   – E-tagging Barbara Block (Day 1 afternoon)

5. Review of genetic studies on population connectivity [rapp- GBYP, Natalia Diaz-Arce]

   – SCRS/P/2023/016 Population connectivity of Atlantic Bluefin Tuna. Naiara Rodriguez-Ezpeleta

6. Reproduction/fecundity at age and identification of spawning sites as it pertains to CKMR parameterization [rapp- GBYP]

   – SCRS/P/2023/014. Brief synopsis of eastern Atlantic bluefin tuna reproductive dynamics. Antonio Medina (Day1)

7. Synthesis of current knowledge on eastern and Mediterranean stock structure (discussion) [rapp- GBYP, Arrizabalaga]

No presentations needed – discussion from previous work

8. Review of genetic methodologies for stock ID, kinship analyses and sex determination. [rapp- GBYP, N. Rodriguez-Ezpeleta]

   - Atlantic bluefin stock structure and Kinship among three larval year classes sampled from Gulf of Mexico and Balearic Islands. Peter Grewe (around 9-11 am CET , Day 2)
   - Methodologies for ABFT Stock ID, kinship, sex determination and others. Natalia Díaz-Arce

9. Review of the potential of epigenetic aging for ABFT [rapp- GBYP, T. Rouyer]

   a. Desk study
      - Assessment of the potential of epigenetic approaches for ageing Atlantic Bluefin tuna samples. Naiara Rodriguez-Ezpeleta. (GBYP Phase 11 report)

   b. Previous studies and presentation of ongoing study
      - Epigenetic Ageing in Fish. Ben Mayne (9-11 am CET, Day 2)

10. Biological sampling [rapp- GBYP, I. Artetxe/P. Lino]

    a. Description of current biological sampling [rapp- GBYP, I. Artetxe]
       a.1 ICCAT GBYP existing programs. Tissue Bank.
          - GBYP biological sampling Francisco Alemany (Day2)
       a.2 CPC sampling programs. EU data collection framework (DCF), others.
          - EU-DCF biological sampling Francisco Alemany (Day 2)
          - Current larval sampling Patricia Reglero
       a.3 Type and place of storage of the biological samples available (EU-DCF Data call).

    b. Future sampling planning [rapp- GBYP, P. Lino]
       b.1 Systematic or target sampling
       b.2 Types of biological samples (calcified structures, gonads, genetic samples, others?). Biological sampling Data Base - Tissue bank.
       b.3 Opportunities for sampling in large numbers. Larvae, juveniles, adults. [larval surveys, ICCAT observers; national observer programs]
          - Larval sampling opportunities Diego Alvarez-Berastegui
          - ROP sampling possibilities. Thomas Franklin (9-11 CET Day 2)

11. Sampling requirements for CKMR [rapp- GBYP

12. Next steps

Appendix 2

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Appendix 3

Background document for discussion on genetic methodologies

Currently, two different genotyping approaches have been specifically developed and applied for kin finding for CKMR in Atlantic Bluefin Tuna (ABFT): DArTCap in the West and an Axiom SNP array in the East. In principle, it might be ideal to use a single approach for all ABFT stocks, but there may also be sound reasons why that would not be worth pursuing. At present, fish genotyped with one approach cannot be directly checked for kinship against fish genotyped with the other, which (although not a deal-breaker for CKMR) might require some samples to be genotyped twice or simply not used. There are cost and reliability implications involved in this, and it is important to decide on the genotyping method (and any adjustments) for eastern-oriented CKMR before a large-scale project begins. The issues should be explored and clarified for GBYP, so that an informed decision can be made.

Although no formal comparisons have been made yet, based on what has been presented to date, it seems likely that:

- Both approaches are adequate for finding kin and for stock ID in both eastern and western samples → comparisons need to be made to confirm whether there is sufficient separation of Full-sibs versus Half-sibs and half-thiatic pair relationships for both techniques.
- The price per sample of both approaches needs to be compared → costs should be provided for each approach.
- DNA quality required for each approach should be assessed.
- DNA cross contamination detection needs to be assessed for both approaches.

Thus, the consequences of using separate methods and of combining methods should be evaluated. A guide is provided below for such an evaluation, with important discussion points identified:

Using a single method would imply either:

1) Running the eastern project based on the DArTcap approach.

   - This will require rerunning some of the samples that have already been genotyped.
     - Is this an issue?
   - All samples/DNA would need to be sent to Australia for genotyping, as only one company (DArT) can apply this method.
     - Is this an issue?
   - The additional information contained in the SNP array will not be used (introgression/adaptation, sex markers)
     - Is this an issue?

2) Running the western samples using the Axiom SNP array.

   - This requires rerunning the samples that have already been genotyped for the West.
     - Is this an issue?
   - This can be carried out in the USA in any genotyping laboratory equipped with a GeneTitan machine (quite standard).
     - Is this an advantage?

3) Merging the loci used in both methods into a single assay.

   - If all loci are to be used, this is the equivalent of having a third method.
     - Is this an issue? - It is an issue because it means that both eastern and western samples will need to be (re)genotyped with this new method.
   - If only a subset of the loci are used for all samples (e.g. western loci), this is equivalent to point 1) above, except for using different methods for genotyping each subset. In other words, some fish would be genotyped using DaRT, while others would be genotyped at the same loci using a newly designed SNP array.

- Is this an issue? - This is a risky option because it is not known how comparable the two methods are (DaRT vs SNP array). Previous comparisons between the SNP method and a similar sequencing method (RAD-seq) suggest that there are biases that can be introduced so that mean genotypes might not be reproducible on the different platforms; this would affect the ability to directly compare genotypes obtained on one platform with those obtained on the other. In addition, the new SNP array would need to be developed. However, this option needs to be evaluated further in order to be 100% certain that it is not possible.

Using separate methods would imply:

1) Samples captured in the West which contain mixed proportions of western and eastern origin samples (e.g., from Canada) cannot be analyzed once and used for both projects. They will need to be analyzed twice if needed for both projects.
   - Is this an issue? -These samples are not strictly needed for CKMR in the EBFT, as such samples can be sourced from East Atlantic and Mediterranean fisheries, which do not reflect mixing with western individuals very much. However, mixed-origin samples (e.g., from the Slope Sea) may be useful to western CKMR in the future. If needed, the cost associated with genotyping some samples twice would need to be calculated, but is expected to be a relatively minor added cost given the numbers of fish genotyped per year for WBFT compared to the numbers required for EBFT.

2) That potential future analyses which require joint analyses of both stocks (e.g., stock merging?) will not be possible unless some of the samples are reanalyzed.
   - Is this an issue?

Run samples in parallel for a time:

A third option is to run WBFT samples in parallel on both genotyping platforms for a period of time, once EBFT becomes operational, so that the two approaches can be directly compared with the intention that the WBFT CKMR could eventually pivot to be incorporated into the EBFT CKMR pipeline. The period of overlap would provide solid proof that information would not be lost should the WBFT study design need to pivot genotyping methods.

- This approach eliminates the need to run the back collections of WBFT, which will constitute a substantial number of samples at the time of implementation of EBFT CKMR (>20,000 fish), while also meeting the goal of a joint study design for all stocks.
- It limits the amount of duplicate genotyping to a couple of thousand fish per year for a limited period of time.