ANNEX 7

19th Meeting of the
International Scientific Committee for Tuna
and Tuna-Like Species in the North Pacific Ocean
Taipei, Taiwan
July 11-15, 2019

SUMMARY REPORT OF THE PACIFIC BLUEFIN TUNA CLOSE-KIN WORKSHOP

July 2019

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Annex 07

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PACIFIC BLUEFIN TUNA CLOSE-KIN WORKSHOP

International Scientific Committee for Tuna and Tuna-Like Species
in the North Pacific Ocean

March 16-17, 2019
Jeju, Korea

1.0 INTRODUCTION

The International Scientific Committee for Tuna and Tuna-like Species in the North Pacific Ocean (ISC) hosted the Pacific Bluefin Tuna (PBF) close-kin workshop in Jeju, Korea 16-17 March 2019. The objectives of the workshop were for ISC members to share progress on close-kin genetics in PBF including sample collection, marker development, and modelling, as well as to discuss ways forward for this collaborative effort to use Close-Kin Mark Recapture (CKMR) as a fishery independent method for estimating PBF population size.

Nineteen scientists from ISC members Japan, Korea, Chinese-Taipei, and USA and two invited experts from Australia attended. Dr. Gerard DiNardo chaired the workshop. The proposed agenda for the meeting was considered and adopted with no changes (Annex 2).

2.0 OVERVIEW OF CLOSE-KIN RESEARCH PROGRAM

G. DiNardo provided an overview of ISC's CKMR research program. This research program is aimed at evaluating the use of CKMR as a tool for developing a fishery-independent estimate of population size of Pacific Bluefin tuna. This method has been used with success in the southern bluefin tuna (SBT), however it has yet to be fully evaluated for PBF.

3.0 REVIEW AND STATUS OF MEMBER COUNTRY CLOSE-KIN PROGRAMS

ISC members Japan, Korea, Chinese-Taipei, Mexico and USA provided overviews of their sample collection to date. Sample sizes compared to targets are summarized in Table 1.

Japan

Japanese researchers have been working on all aspects of CKMR including sample collection, modeling, and development of genotyping methods. Samples are being collected through port sampling, working with fish buyers, and dedicated young of year (YOY) sampling efforts. A tally of samples collected in 2016 and 2017 are provided in Table 1. Samples from 2018 are still being tallied but are lower in number from previous years due to management restrictions. A

proportion of the overall genetic samples have otoliths and vertebrae associated with them to help in determining age and spawning ground origin. Much of the current work is focused on the development of genotyping methods and modeling with the first goal being an estimation of population size through half-sib pair (HSP) analyses. A more detailed discussion of these topics was had in later talks.

Korea

Korean researchers have been actively collecting samples from their fisheries and developing markers for genotyping. Samples collected during the period of 2016-2018 are presented in Table 1. So far 288 samples have been collected in 2019. The age structure of collections appears to be mostly ages 0-3 and sample sizes in recent years have been reduced due to fishery restrictions. Effort has been put into development of microsatellite loci for use in genotyping through a combination of new nextgen sequencing data and data mined from public data repositories. A final set of 33 microsatellite loci have been identified that can be used for genotyping.

Chinese-Taipei

Taiwanese researchers have been actively collecting samples and developing genotyping methods. Overall sample size for 2016-2018 are presented in Table 1. Depending on the sampling year 38.5-64.2% of these samples have associated otoliths. The age classes represented are primarily ages 6-15 but range to 25. Effort has been put into development of SNP genotyping methods using a variety of techniques including ddRAD, DArTcap, and targeted Ampliseq. Unfortunately it seems that many of these samples have degraded DNA which had led to disappointing results from the ddRAD based methods. However, the targeted Ampliseq method has had good success with 89.5-97% success in genotyping. Further work is needed to identify additional loci for a method such as Ampliseq that is robust to poor sample quality.

Mexico

Mexico has been focused entirely on sample collection and has not conducted genotyping work. The samples collected are in collaboration with the tuna ranch operators at harvest time so are sampled after 1 year in captivity. Overall sample sizes for the years 2015-2018 are presented in Table 1. The majority of these fish are age 2-3 at the time of capture. Sampling is expected to continue with a set annual goal of 750 samples. No otoliths were collected from these samples.

USA

American researchers have been focused on sample collection and development of methods to identify natal origin based on otolith microchemistry but have not conducted any genotyping. Samples have been collected from dockside sampling, recreational fishery processors, and from the purse seine fishery with 4168 samples available from 2011 to present with larger annual samples collected beginning in 2016. A subset of samples representing only the 2015, 2016, and

2017 cohorts (n=1660) are reported in Table 1. For the 2016-2018 samples 38-62% of the samples have associated otoliths. A collaborative project with Texas A&M University is underway to use otolith microchemistry of the otolith cores to assign natal origin. There is high discrimination succcess using this method (~90%) but the signal varies annually.

Table 1. Pacific bluefin tuna (*Thunnus orientalis*) tissue samples collected by ISC member countries to date.

Fishery Area	**Sampling years**	**Age Class**	**Countries**	**Suggested sample size**	**Samples collected**	**notes**
East Pacific	2016, 2017, 2018	Age 1-3	USA, Mexico	1300	3660+	Includes only the 2015-18 cohorts from the US and all of the Mexico samples
West Pacific	2016, 2017, 2018	Age 1-3	Japan, Korea	1300	1638	Just Korea (includes some age 0), not clear if Japan sampled this age group
West Pacific	2016, 2017, 2018	Age 0	Japan	1300	2121	
West Pacific (Sea of Japan)	2015, 2016, 2017	Age 3+	Japan	1680		Not clear from presentation whether fish came from Sea of Japan or Okinawa-Taiwan area or both
West Pacific (Okinawa-Taiwan area)	2015, 2016, 2017	Age 3+	Chinese-Taipei, Japan	2220	11470+	Not clear from presentation whether fish came from Sea of Japan or Okinawa-Taiwan area or both

4.0 ADVANCES IN CLOSE-KIN MARK RECAPTURE RESEARCH

M. Bravington provided an overview of CKMR research and recent advances. Much of the theory can be found in the Bravington et al. 2016 Statistical Science Paper, "Close-Kin Mark-Recapture." Parent offspring pairs (POPs) and half-sibling pairs (HSPs) can reliably be detected from genetics; the rest is just math and logistics. He described CKMR models and how to work out kinship probabilities or expected relative reproductive output (ERRO) as well as how to fit a CKMR model. POPs provide information about relative fecundity at age and total reproductive output but it is important to understand beforehand the effects of relative fecundity. However, they don't reveal the number and age of fish that the total reproductive output came from (e.g. from 1 million 3 year olds or 100,000 13 year olds). HSPs provide some estimates for adult abundance, but do not provide direct estimates of abundance unless relative fecundity is minimal (e.g. sharks and marine mammals). HSPs come with some caveats, comparisons within a cohort are problematic due to potential sweepstakes recruitment issues, similarly HSPs are of the same relational score as grandparent-grandchild, half cousins, etc so it is important to account for these potential interfering relational categories. POPs and HSPs are good complements and provide a good picture of population dynamics for adults, using both is recommended. For CKMR of non-spatial teleosts the following are needed: adults across the full size/age range, juveniles of known ages across several cohorts, POPs, HSPs, adult sex and age (good growth curves can help get appx ages). The essential CKMR steps were described as was where CSIRO is in the process. It was noted that the R package "Kinference" will use SNP data and should be released in 2019. It will identify POPs, HSPs, and FSPs, as well as do some quality control. An in-house software package "genocalldart" is available to aid in discriminating single-null vs. homozygote SNP data calls.

For CKMR, CSIRO currently uses the Diversity Arrays Technology (DArT) capture array technology to identify SNPs at a current cost of ~ $15 AUD each, not including DNA extraction. This is the second generation of markers used for this method with microsatellites initially used for CKMR application in SBT. The DArTcap technology has been applied to SBT as well as a number of other species at this point.

5.0 STATUS AND NEXT STEPS OF THE CCSBT CLOSE-KIN RESEARCH PROGRAM.

M. Bravington also provided an update regarding SBT close-kin work. CSIRO is using CKMR both as a stand alone abundance estimate and incorporating it into the assessment. They routinely score 2000 SNPs, using 1500 loci for downstream analyses. They are able to use read depth as a tool to identify the presence of null alleles at individual loci. At present, they genotype ~2000 SBT per year at a cost of $100k AUD. This cost does not include the initial costs for locus development and research.

6.0 JAPAN TECHNICAL PRESENTATIONS

6.1 Sampling design for modelling

Y. Tsukahara presented the recommended sampling design from the perspective of a modeling framework. For example, the possible compared pairs should be pairs from samples whose spawning and spawned grounds are the same in the case of POPs. For that point, tissue sampling along with the otolith collection for age determination and the vertebrae that are useful for determination of spawning ground is recommended. The presenter also mentioned the qualities of collected samples in Japan. Five percent of the samples were of insufficient quality for genotyping.

6.2 Genotyping method

A. Suda presented genotyping methods. There were four parts in the talk: (1) an improved draft genome of PBF, (2) re-sequencing data collection, (3) Custom Ampliseq SNP panels and (4) Random Ampliseq SNP panels. The new genome coupled with the resequencing data (61 individuals across both spawning grounds) provide a wealth of data on genetic variability along with identification of a sex-linked marker for genetic sex determination. The resequencing effort resulted in ~25x coverage and identified over 17 million SNPS. A custom Ampliseq SNP panel was developed to genotype 200-300 SNPs for POP comparisons, this panel includes 3 sex loci and has a low error rate (2-5%). For analyses requiring identification of both POPs and HSPs a random Ampliseq method was applied generating 3000-4000 SNPs with 99% accuracy. These genotyping methods appear to be highly accurate and are expected to collect enough SNPs for CKMR analysis for both POP's and HSP's. The random Ampliseq method has been applied to 500 2016 and 500 2017 young of year to begin to look at application of the markers for HSP identification.

6.3 Kin recognition

R. Nakamichi presented the methodologies of kin recognition using SNPs. Basic exclusion method for POP recognition, likelihood odds ratio method for POP/HSP recognition, and 2D plotting of IBD (identity by descent) for general types of kin recognition were implemented. When finding target kin (POP/HSP) using actual data, other types of kin (grandparent-offspring, uncle-nephew, cousin, etc.) overlap on HSP and make the recognition difficult. Considering this problem, simulated data analysis showed that, given a minor allele frequency greater than 0.3, ~120 SNPs are required for POP recognition and ~3,000 SNPs are required to fully resolve HSP recognition.

6.4 Individual based model

T. Akita presented an approach for using an individual-based model and introduced software to handle it, which can generate a kinship relationship in both population and sample and thus play a role of "operating model" for CKMR. Advantages of the current software are to handle i)

complex population structure, ii) realistic population size, such as 10s of millions, iii) flexible setting of reproduction, including "lucky-litter" and "faithful marriage" effects, and iv) to generate complex kinships and SNPs patterns. Under the assumption of a current view of PBF ecology (two-spawning grounds), the kinship pattern in the sample was calculated. Results showed that many types of kinship pairs are found which are potentially mis-classified as HSPs, such as half-uncle/nephew, half cousin, and half-grand uncle/nephew, and the number of those pairs were more than twice that of HSPs, providing material for further discussion to the methodology for PBF-CKMR, especially the number of genetic markers and their resolution.

7.0 TISSUE PROCESSING STRATEGIES

7.1 Methodologies and protocols.

P. Grewe presented on strategies for high throughput genotyping CKMR and genetagging. CSIRO has devoted a significant amount of effort towards improving sampling and processing efficiency while at the same time reducing the risk of cross contamination between fish. This work has resulted in the creation of a low-cost single use biopsy sampler. In addition to streamlining the field collection of samples, the use of high-throughput robotics and standardized extraction protocols has allowed a more streamlined and efficient processing of samples. A discussion was had on the evolution of genotyping from the original 25 microsatellite loci to the current use of ~2000 SNP loci using the DArTcap technology. They are employing this technology in SBT for both CKMR as well as genetagging of juveniles (60-90 SNP loci) to produce estimates of juvenile abundance. Some discussion was had on the need for QC methods (e.g. excess heterozygosity) to screen for cross contamination.

7.2 ISC Member Work Plans and Timelines

Most member countries have thus far focused on collection of specimens. Japan has begun developing methods for identification of appropriate genetic markers using Ampliseq approaches, and these data have not yet been made available to the broader ISC community. The scientists hope to publish preliminary methods/markers by Custom Ampliseq approach developed in Japan which can then be shared within one year. This marker panel can be detecting tools for POPs and comparable to it developed in Taiwan. The Custom Ampliseq approach is expected that generated data by this method can be shared among labs and facilities. Other member countries have not provided time lines for genetic analysis.

A discussion was had regarding the overall work plan for CKMR with HSPs and how each member country would be able to generate and disseminate data. It was discussed that there were only two viable options for to reach the project's goal of estimating the PBF population size with both POPs and HSPs, given each country's progress and in-country capacity for lab work and the

generation of data. Both of these options required samples to be processed at a single facility at either a third-party site (e.g., DArT Australia) or Japan. It was also noted that exploration of POPs using Custom Ampliseq in each country's facility will be informative for further understanding of the stock structure genetically.

It was noted that Japan has invested significant resources into marker development and thus preferred to continue processing their own samples. Using a third-party was not considered as an option and they are not capable of processing larger numbers of samples at the current time. Thus Japan was not an option as a central processing facility for the project.

Taiwan scientists indicated that they could send samples to either Australia or Japan, but that due to sample quality issues the current samples are likely only of use using amplification based methods. As with Japan, they are limited by their capacity to process large numbers of samples so could not be a central processing facility, however they are able to process their own samples. Taiwan will publish their ampliseq marker data in the near future.

Mexico could send samples overseas but wanted to verify before committing as well as assess capacity for what could be done in-country (e.g., DNA extraction) and what should be sent out.

For Korea, current regulations on shipping samples internationally need to be verified. It may be possible to use a Korean company to process samples. They proposed combining Ampliseq data across labs which may be better than the DArTcap procedure.

8.0 INCORPORATING CLOSE-KIN DATA INTO STOCK ASSESSMENTS

8.1. Pacific Bluefin Tuna, Workplan, Timeline

An overall timeline and potential work plan were not established. Given the constraints on marker data and sibling pair availability, the participants agreed that the best way forward was for each country to perform DNA extraction on samples collected to assess DNA quality and wait for genotyping methodology to be published for future analysis. It will likely be several years before an abundance estimate based on CKMR will be available.

ANNEX 1
Participants list

Australia

Mark Bravington
CSIRO
Mark.Bravington@data61.csiro.au

Peter Grewe
CSIRO
Peter.Grewe@csiro.au

Japan

Tetsuya Akita
National Research Institute of Fisheries Science
akitatetsuya1981@affrc.go.jp

Hiroaki Okamoto
NRIFSF
okamoto@affrc.go.jp

Hiromu Fukuda
NRIFSF
fukudahiromu@affrc.go.jp

Ayako Suda
NRIFSF
ayasuda@affrc.go.jp

Reiichiro Nakamichi
National Research Institute of Fisheries Science
nakamichi@affrc.go.jp

Yosuke Tanaka
NRIFSF
yosuket@affrc.go.jp

Yohei Tsukahara
NRIFSF
tsukahara_y@affrc.go.jp

Shuya Nakatsuka
NRIFSF
snakatsuka@affrc.go.jp

Mexico

Michel Dreyfus Leon
INAPESCA
dreyfus@cicese.mx

Republic of Korea

Doo Nam Kim
NIFS
doonam@korea.kr

Mi Kyung Lee
NIFS
ccmklee@korea.kr

Eun Mi Kim
NIFS
ocean0629@korea.kr

Sung Il Lee
NIFS
k.sungillee@gmail.com

Chinese-Taipei

Wei-Jen Chen
National Taiwan University
wjchen.actinops@gmail.com

USA

Matthew Craig
NOAA
matt.craig@noaa.gov,

John Hyde
NOAA
john.hyde@noaa.gov

Gerard Dinardo
NOAA
gerard.dinardo@noaa.gov

ANNEX 2

Agenda

International Scientific Committee for Tuna and Tuna-like Species of the North Pacific Ocean (ISC)

PBF Close-Kin Workshop

March 16-17, 2019
Maison Glad Jeju Hotel
Jeju, Korea

1. Welcome
2. Introductions
3. Goals and Expectations
4. Review and Adoption of Agenda
5. Overview of ISC Close-Kin Research Program
6. Review and Status of Member Country Close-Kin Programs
    a. Japan
    b. Korea
    c. Taiwan
    d. Mexico
    e. USA
7. Status and Next Steps of CCSBT Close-Kin Research Program
8. Tissue Processing Strategies – Open Discussion
    a. Methodologies and protocols
    b. Workplan and timelines
9. Incorporating Close-Kin Data into Stock Assessments
    a. CCSBT
        i. status, workplan, and timeline
    b. PBF
        i. activities, workplan, and timeline
10. Open Discussion