Commission for the Conservation of
Southern Bluefin Tuna

みなみまぐろ保存委員会

CCSBT–CC/1410/17

Review of Current R&D Technological Developments and Tools Available to Assist Certifiers and Validators to Identify SBT

1. INTRODUCTION & BACKGROUND

1.1 Introduction

An important element of the current CCSBT Compliance Action Plan (CAP) is the continued monitoring of research and development initiatives that could be used to help achieve improved Southern Bluefin Tuna (SBT) compliance monitoring.

As part of this monitoring, the Eighth Meeting of the Compliance Committee (CC8) tasked the Secretariat with developing a review/summary of current R&D technological developments and tools available to assist certifiers and validators to identify Southern Bluefin Tuna (SBT).

1.2 Background Information from Previous CC Meetings

Since the Seventh Meeting of the Compliance Committee (CC), CC agendas have included the standing item, 'Research and development of new technologies & tools to aid observers, certifiers and validators to identify SBT, in particular once processed'. Under this item, Members have been asked to prepare and present specific proposals for consideration by the CC so that recommendations can be made to the Extended Commission (EC) regarding support and/or funding of any proposed projects as appropriate.

At CC7, Japan noted that other Regional Fisheries Management Organisations (RFMOs) are investigating methods for traceability of fish products and that this could be a new technology that should be reviewed by future CC meetings.

At CC8, it was noted that there was work underway in several countries to help identify SBT including DNA testing, skin analysis, electrophoresis, and other genetic species identification work. New Zealand commented that it was working on developing genetic probes as well as a portable testing device to avoid the requirement for laboratory testing. In addition, Australia presented a brief summary describing the gene tagging work it was involved with.

1.3 The Secretariat's Review

The Secretariat has broadly reviewed the available research on tuna identification/ tools, including contacting some Members about their most recent research activities in this area. This review is not exhaustive. It focuses on genetic species identification methods, and takes the approach of first summarising the types of tools available, then comments on their potential use by field personnel.

A summary of relevant Member-specific and international research, including some traceability projects, is included in section 3.

2. SECRETARIAT REVIEW

2.1 Overview of Genetic Identification Tools

There are eight species of tuna in the genus *Thunnus*. Identification of these species is difficult given their close genetic relationships and the ease with which distinguishing morphological characters can be removed once landed (Bartlett and Davidson, 1991). Therefore, tools that would enable rapid and reliable identification of tuna to species level in the field are potentially very important for the effective monitoring of tuna traded in various raw and/or more processed forms, and for the detection of Illegal, Unregulated and Unreported (IUU) fishing activities in general.

Techniques for the genetic testing and identification of tuna species from tissue samples have already been developed and utilised by scientists around the world.

In terms of CCSBT's compliance objectives, the main issue associated with most of these tools is that they require complex and expert laboratory analyses of the tissue samples collected over a period of days/weeks. Therefore, most of the tools already available could not currently be utilised by CCSBT certifiers or validators for real-time analysis in the field.

However, the Secretariat notes that recent developments in so-called 'real time PCR[1] techniques' appear to provide the first real potential for development of reliable and practical identification tools that could potentially be used by field personnel such as certifiers and validators in order to verify tuna species.

2.2 DNA Barcodes and the '*COx*I' Marker

DNA barcodes are a global standard for species identification, and the standard marker traditionally used for DNA barcoding of many animal species including (*Thunnus*) species identification is cytochrome oxidase subunit I (*COx*I). The Barcode of Life Database (BOLD) holds genetic barcode data on the *COx*I marker for many different fish species. Sequencing of this region of mitochondrial DNA (mtDNA) from a fish species provides relatively accurate information on species identification.

Disadvantages

However, use of BOLD *COx*I genetic markers alone do not necessarily allow full discrimination of all eight tuna species from each other (*e.g.* Lowenstein, Amato and Kolokotronis, 2009).

Other disadvantages of more traditional DNA 'barcoding' techniques include:

- Limited ability to reliably identify tuna to species level,
- Non-portability and time delays and large costs associated with conducting the required laboratory analyses, and
- Limited or no ability to reliably test samples where the DNA has been degraded, *e.g.* in archival and/or highly processed products such as canned tuna.

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[1] Polymerase Chain Reaction (PCR) uses primers to amplify specific DNA fragments from tissue samples. The amplified DNA fragments can be used for laboratory-based genetic identification purposes (*e.g.* for sequencing).

2.3 PCR Fragment Sequencing Methods

There is now a suite of sequencing methods (that essentially utilise mini-barcodes) emerging that may alleviate some of the issues associated with the more traditional barcoding techniques. Some of these methodologies may soon provide the facility to conduct large-scale reliable 'real-time' in the field identification of tuna species, either relatively unprocessed or in highly processed seafood products at a much lower cost than previously possible.

Several types of these methodologies are described below.

2.3.1 PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)

Takeyama *et al.* (2001) demonstrated that all tuna species could be distinguished using PCR-RFLP, but five different restriction enzymes needed to be used to achieve these results.

Lin, Shiau and Hwang (2005) used sequence analysis and PCR-RFLP plus three restriction enzymes to facilitate rapid identification of 4 common *Thunnus* species – *T. thynnus* (Atlantic bluefin tuna - ABT), *T. alalunga* (albacore - ALB), *T. obesus*, (bigeye tuna - BET) and *T. albacares* (yellowfin tuna - YFT) collected at domestic and foreign ports. The same technique was used to successfully determine the tuna species of twelve samples of commercial tuna fillets (sashimi) purchased from six different Taiwanese markets.

2.3.2 PCR - Forensically Informative Nucleotide Sequencing (PCR-FINS)

FINS is a PCR and DNA-sequencing method that can be used for identification of processed or un-processed tissue samples. It has been described as the most accurate technology for tuna species authentication (Chuang, Chen and Shiao, 2012). This methodology was used in the analyses conducted by Viñas and Tudela (2009), Botti and Giuffra (2010), and Tseng, Shiao and Hung (2011).

Viñas and Tudela (2009) validated a FINS methodology that identified all eight *Thunnus* species (including SBT) from any type of tissue including sushi and sashimi. This methodology is the one that was used by the World Wildlife Fund (WWF) to test sashimi-grade tuna in supermarkets, restaurants and import facilities in China during 2011 and 2012, the results of which were reported to CC7.

Botti and Giuffra (2010) used an existing PCR-FINS methodology to detect mitochondrial polymorphisms in food samples. As the methodology used was largely independent of the degree of degradation of the DNA source (*e.g.* by cooking, processing and/or auto-claving), it could be applied to processed seafood. This study facilitated the identification of seventeen fish species within the Scombridae family, including all eight tuna species, from canned tuna, tuna salad and tuna sauce samples.

Tseng, Shiao and Hung (2011) also used a PCR-FINS methodology to distinguish the three morphologically similar species of bluefin tunas: *T. orientalis* (Pacific bluefin tuna - PBT), SBT and ABT.

However, disadvantages of PCR-FINS methodologies include the relatively higher costs and longer time periods required for DNA sequencing (days in the laboratory), which makes them unsuitable for high-volume identification work (Chuang, Chen and Shiao, 2012). These characteristics would also make them unsuitable for real-time analysis of samples in the field by personnel such as certifiers or validators at this point in time. However, technology is advancing rapidly in this area and it's possible

that much more time-efficient analysis techniques could become available relatively soon.

2.3.3 Real-Time PCR Techniques

More recently, real-time PCR techniques have been developed. The main advantages of these real-time PCR techniques are that they possess characteristics of high-sensitivity, high-specificity and excellent efficiency (one step). In addition, because there are no post-PCR steps, the risks of cross-contamination are decreased (Chuang, Chen and Shiao, 2012). Therefore, they provide the best opportunity yet for potential development into portable, efficient tools that could be used by field personnel such as certifiers or validators in the future.

Chuang, Chen and Shiao (2012) successfully used two real-time, one-step PCR techniques for the rapid identification of four tuna species: BET, PBT, SBT and YFT. Both techniques could distinguish the four tuna species in canned products in an efficient manner at high-volume. The whole procedure, including DNA extraction significantly reduced the experimental time required to within half a day. This smaller processing and analysis timeframe facilitates the efficient utilisation of these methods on a larger, more commercial scale.

3. RESEARCH PROJECTS

3.1 Australia

Australian scientists have been focusing on developing real-time PCR techniques or so-called "Lab-on-a-chip" technology, *i.e.* sampling tools that are robust, reliable, tamper-proof and cost-effective enough for ease of large-scale implementation of gene tagging in the field. These tools utilise a suite of Single Nucleotide Polymorphisms (SNP) markers and are specifically designed for high-volume forensic grade identification purposes. Much of the genetic marker work for SBT gene tags has been done in cooperation with the genetic research required for the Close-Kin (C-K) abundance estimate project.

SNPs and DNA microsatellites provide a DNA profile or gene tag enabling researchers to:

- Distinguish SBT from other tuna species, and/or
- Identify specific SBT individuals – for example portions of processed SBT could feasibly be matched back to the whole (or less processed) SBT individual at an earlier part of the supply chain.

Cost Efficiencies

As mentioned in 2.3.3, the advantage of these new genetic sampling tools is that less expertise is required in sampling, tissue handling and genetic profiling than earlier molecular techniques. A portable testing device can be used, thereby removing the need for expert laboratory testing. Costs involved are therefore also significantly reduced.

Research Progress

Considerable research progress has already been made on this research over the past twelve months. Well-developed genetic species identification markers are now available for four tuna species: SBT, BET, YFT and skipjack (*Katsuwonus pelamis - SKP*). Similar markers will soon be available available for identifying Atlantic Bluefin tuna (ABT), Northern Bluefin (NBT), longtail, and blackfin tunas as well.

Another independent project aims to demonstrate that genetics techniques can be used for 'real- time' species identification of sashimi-grade species in the field. This project should be completed by the end of 2014.

3.2 European Union
FishPopTrace[2] Traceability Project
FishPopTrace is a project that was launched in March 2008 under the umbrella of the EU Seventh Framework Programme (FP7), and was concluded in July 2011. It focused on four important EU commercial fisheries: cod, hake, herring and sole. Its aim was to construct a Pan-European framework for product traceability and policy related monitoring, control and surveillance (MCS) in the fisheries sector based on advanced technologies. It involved fifteen research groups (from the EU, Norway and Russia), specialising in fish population genetics, molecular biology, proteomics, microchemistry and biochemistry, experts in wildlife forensics, stakeholders of the fisheries industry and a US National Oceanic and Atmospheric Administration (NOAA) scientific consultant.

3.3 Japan
Japan did not provide any specific research updates to the Secretariat, however the Secretariat notes the Takeyama *et al*. (2001) paper included in this review, as well as the recent research of Nakamura *et al*. (2013) who have determined the genome sequence of Pacific Bluefin tuna (PBT) using next generation sequencing technology.
In addition, in its National report to CC9, Japan indicated that it undertook some genetic testing of domestic bigeye tuna samples for verification purposes during the 2014/15 fishing season.

3.4 New Zealand
New Zealand has provided the following updated information to the Secretariat regarding a research project that has just commenced.

In August 2014, New Zealand's Ministry of Primary Industries (MPI) contracted a research provider to provide a user-friendly, scientifically sound tool or method capable of accurately identifying fresh/frozen tuna (genus *Thunnus*) trunks to species level. It was specified that the tool/method should be easily deployable in the field, environmentally robust and cost effective, and ideally have the following characteristics:

- Comparable specificity and sensitivity with gold standard (DNA sequencing)
- Based on internationally recognised underpinning science
- Field deployable
- Able to be utilised effectively in harsh environments *e.g.* at sea, low temperatures, low ambient light
- Applicable to frozen tuna trunks
- Easily validated for use – high level of repeatability, reproducibility and statistically robust
- Real time identification
- Low operating cost
- Low long term maintenance costs.

[2] https://fishpoptrace.jrc.ec.europa.eu/home

Progress
To date, the following progress has been made:

- Muscle tissue samples have been collected from five albacore and five SBT
- Albacore samples were collected at Mooloolaba, Sunshine Coast, from fish that were caught at sea about 12-16 hours prior to sample collection. The fish were kept in an ice slurry until processing
- Southern Bluefin tuna samples were collected in Port Lincoln, within a few hours of being harvested
- RNA was extracted from these ten samples (5 samples per species), analysed using 'bioanalyser' to determine RNA quality, and samples were then sent to an Australian genome research facility
- Pacific Bluefin tuna (PBT) muscle samples have not yet been collected, however in the interim scientists will compare potential distinct epitope sites with the published PBT genome sequence.

3.5 Taiwan
The Secretariat understands that research is likely to be continuing in Taiwan, and notes the three papers already referred to in this review:

- Lin, Shiau and Hwang (2005),
- Tseng, Shiao and Hung (2011)
- Chuang, Chen and Shiao (2012).

3.6 GEF[3] Project: Sustainable Management of Tuna Fisheries and Biodiversity Conservation in the Areas Beyond National Jurisdiction (ABNJ)
As part of this current GEF funded project, a specific activity/sub-project has been defined which aims "to establish best practices for traceability and legal provenance CDS systems for tuna fisheries". This sub-project could potentially provide recommendations about best practice tools (including genetic identification tools), that certifiers and validators could use to reliably verify/identify different tuna species in the future. The CCSBT Secretariat will be providing input to this project activity during late 2014.

3.7 The Fish Barcode of Life Initiative (FishBol[4])
The Fish Barcode of Life Initiative is a global effort to coordinate an assembly of DNA barcodes, images and geospatial coordinates for all fish species. Some of the aims of this project are to facilitate fish species identification for all potential users, and to enable identifications where traditional methods may not be applicable. This initiative runs in partnership with the Consortium for the Barcode of Life (CBOL) and Census of Marine Life Projects.

[3] Global Environment Facility

[4] http://www.fishbol.org/

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