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# Update on the SBT close-kin tissue sampling, processing, kin finding and long-term sample storage.

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# Contents

# 1 Abstract

Muscle tissue samples were collected from SBT landed by the Indonesian longline fishery in Bali, Indonesia (adults; n=1500) and from harvested SBT at tuna processors in Port Lincoln, Australia (juveniles; n=1600) in 2017/18. Samples collected in Indonesia are stored at -20°C at the RIMF facility during the harvest season (Sep-Apr). They will be transported frozen to Hobart and held at -20°C until they are processed.

Muscle samples from the 2016/17 season were subsampled and the DNA subsequently extracted. A portion of the DNA was sent to DArT for genotype sequencing. The remaining tissue and extracted DNA samples were moved to -80°C where they currently remain.

DNA extracts from the 2015/16 muscle tissue samples selected for genotyping (Farley et al. 2017) were processed by DArT and the genotype data sent to CSIRO in October 2017. The kin-finding analyses to identify parent-offspring pairs (POPs) and half-sibling pairs (HSPs) were updated to include these data, and the identified POPs and HSPs were provided to the CCSBT in April 2018. Note, however, that the DArTcap data added to the analysis this year was not entirely consistent with the previous data. We are still investigating why this was the case, and what the implications might be for ongoing kin finding in the future. There may need to be further modifications to the genotyping and/or analytical and processes to improve quality control and consistency. The outcomes of the further investigations will be reported in 2019.

Assuming, the CKMR (and gene tagging) projects remain part of the ongoing monitoring of the stock, long term storage needs for archived tissue and extracted DNA samples must to be considered. Ultra low temperature freezer space (and space to house freezers) at CSIRO is a finite resource, and as far as we are aware, there are no commercial facilities that store samples at -80℃ in Hobart. Investigation of alternatives storage solutions and maximum retention time for samples are recommended.

# 2 Introduction

In 2013, the Extended Scientific Committee (ESC) developed a new Scientific Research Plan for southern bluefin tuna (SBT). The SRP was reviewed in 2014 and again in 2015. Several items were identified as high priority in the work plan including the continued collection and genotyping of tissue samples for ‘close-kin mark recapture’ genetics to assess the abundance of adult southern bluefin tuna (SBT). The CCSBT has funded the collection and archiving of SBT muscle tissue (since the 2014/15 season) and DNA extraction & sequencing of the tissue samples (since the 2015/16 season). In 2018, the CCSBT also funded the analysis of the sequencing data to find parent-offspring and half-sibling pairs in the samples (close kin identification). Table 1 shows the work undertaken in each project since 2015. In this paper we provide an update on progress of activities in 2018. We also review long term storage options for tissue and extracted DNA samples.

**Table 1. Summary of SBT close-kin work undertaken each year since 2015. For the genotyping and kin-finding analysis, the season in which the fish were sampled is given.**

| Project | Muscle tissue collection | DNA extraction & genotyping | Close kin finding | ESC paper |
|---|---|---|---|---|
| **2015** | 2014/15 | NA[1] | NA[1] | CCSBT-ESC/1509/15 |
| **2016** | 2015/16 | 2014/15 | NA[1] | CCSBT-ESC/1609/08 |
| **2017** | 2016/17 | 2015/16 | NA[1] | CCSBT-ESC/1708/09 |
| **2018 (current project)** | 2017/18 | 2016/17 | 2015/16 | Current paper |

[1] Genotyping & close kin finding undertaken in FRDC project 2016-044 (see Bravington et al. 2017).

# 3 Muscle tissue collection

In Indonesia, targeted sampling of SBT occurred at Benoa Fishing Port in the 2017/18 spawning season using the existing Indonesia-CSIRO monitoring system for the longline fishery (e.g. see Proctor et al, 2006). Length measurements and muscle tissue samples were obtained for 1500 SBT ranging from 134-209 cm fork length (FL). These are the same fish also sampled for otoliths (see Sulistyaningsih et al, 2018).

In Australia, muscle tissue samples were collected from juvenile SBT in June-July 2018 at the tuna processors during harvest in Port Lincoln, South Australia. Tissue was obtained from 1600 fish ranging from 98 to 109 cm FL to endure the full size range of 3 year-olds is being sampled. The muscle tissue was frozen according to protocols provided by CSIRO.

The muscle samples are stored frozen in consecutively labelled boxes with 100 positions (10 by 10) in each box (A01 through J10). Individual sample are given a unique identification label (e.g., SbPL2014_Bx01_A01) and will be stored in -80°C freezers.

# 4 Close kin genotyping

A total of 2024 muscle tissue samples collected in the 2016/17 season were selected for genotyping. Of these, 1012 were from fish caught by the Australian surface fishery in the Great Australian Bight (juveniles) and 1012 from fish caught by the Indonesian longline fishery and landed in Bali, Indonesia (adults).

The samples from Australia were selected for analysis based on fish length. Of the 1012 selected, all samples from lengths 98-106 cm FL were selected and 100 and 53 were randomly selected from the 107 and 108 cm length classes (Figure 1). This selection process reduced the chance that 4 yo fish were selected for analysis.

The samples from Indonesia were selected based on size of fish. Only fish ≥150 cm FL were included to avoid potential sampling of immature fish. All large fish ≥175cm were selected plus

additional fish (up to a total sample size of 1100 fish) were selected randomly within each of the remaining 1-cm length class (150 -174 cm).

DNA was extracted from a 10mg sub-sample of tissue from all fish. For most samples, a magnetic bead-based extraction protocol (Machery Nagel Nucleomag) kit was used on an Eppendorf EP motion robot to produce a 150uL archive and 50uL working stock of DNA in micro-titre format plates.

Archive plates of extracted DNA are stored in dedicated -80'C freezers located at CSIRO Hobart. Working stock plates of extracted DNA were shipped to Diversity Arrays Technology (DArT) in Canberra for genotype sequencing, referred to as "DArTcap", of approximately 2000 single nucleotide polymorphic loci (SNPs). When completed, the sequencing information will be transmitted to CSIRO Hobart.

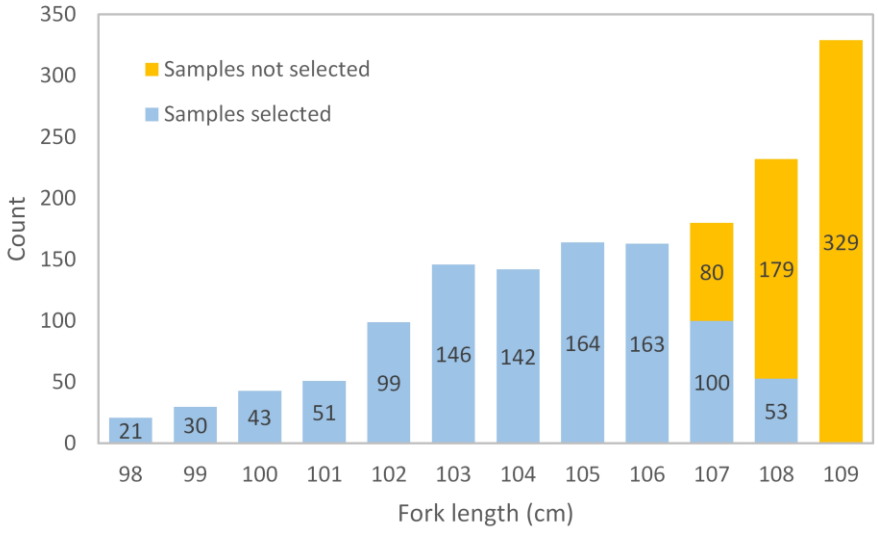


**Figure 1. Length frequency of SBT selected for close-kin mark recapture genotyping from the 2017/18 samples collected in Australia Port Lincoln.**

# 5 Close kin finding

DNA extracts from the 2015/16 muscle tissue samples selected for genotyping (Farley et al. 2017) were processed by DArT and the genotype data sent to CSIRO in October 2017. The kin-finding analysis database used for identification of parent-offspring pairs (POPs) and half-sibling pairs (HSPs) was updated to include the 2015/16 data.

Prior to kin-finding, we had to "call the genotype" for each fish and locus in the new data (i.e., use the sequencing data to infer the pair of alleles present). This genotype-calling entails quite complicated algorithms developed at CSIRO specifically for DArTcap sequencing data, and also estimates the genotyping error-rates for each locus, which is important in the identification of half-sibling pairs. Kin-finding was then carried out on the entire dataset of genotypes.

Similar to last year, a series of quality control (QC) steps were applied to the data to remove fish with unreliable genotype calls; this includes a test of whether a fish has an unexpectedly high

number of heterozygous loci, which could be an indication of cross DNA contamination between individuals. An additional QC step (which is a more stringent heterozygosity test) was applied to the data for HSP-finding than for POP-finding, since the statistic used to identify HSPs is more sensitive to poor quality data. The QC steps used this year were slightly different than in 2017, so they were applied to the entire dataset, not just the new data. After applying the QC steps, 17,467 fish (adults and juveniles) remained for POP-finding (out of the original total of 19,322), and 11,928 juveniles for HSP-finding (Table 2).

**Table 2. Number of fish used in POP-finding and HSP-finding after quality control (QC) checks were applied. Note that an extra QC check was applied to the juveniles for HSP-finding relative to the 2017 analysis reported in Bravington et al 2017. Note that for the adults, samples were collected from Indonesia in the fishing season ending in the year shown (i.e., samples collected over the 2005/06 fishing season are referred to as year 2006).**

| | POP-finding | | HSP-finding |
|---|---|---|---|
| Year | Adults | Juveniles | Juveniles |
| **2006** | 0 | 1323 | 1297 |
| **2007** | 0 | 1328 | 1306 |
| **2008** | 0 | 1360 | 1332 |
| **2009** | 0 | 1350 | 1331 |
| **2010** | 976 | 1320 | 1295 |
| **2011** | 962 | 966 | 950 |
| **2012** | 536 | 883 | 857 |
| **2013** | 965 | 906 | 889 |
| **2014** | 923 | 900 | 889 |
| **2015** | 0 | 955 | 938 |
| **2016** | 957 | 857 | 844 |
| **Total** | 5319 | 12148 | 11928 |

## 5.1 POP-finding

We used the genotype data to identify POPs using the same method as in 2017, which is a modified Mendelian-exclusion statistic referred to as the Weighted-PSeudo-EXclusion (WPSEX) statistic (see Appendix B of Bravington et al. 2017). Figure 1 shows part of the histogram of the WPSEX statistic, across all genotyped adult-juvenile pairs (12148 juveniles x 5319 adults = 64.6 million comparisons). The POPs are visible as a small bump on the left side, and are clearly separated from non-POPs. Most of the histogram (to the right) has been truncated, because otherwise the POPs are too few compared to the gigantic bump of unrelated pairs (whose peak is around 0.116 – exactly where theory predicts it should be based on allele frequencies of each locus) and could not be visualized. The giant bump drops off very quickly to the left of ~0.08, and the flattish tail around 0.055-0.075 will contain a number of adult/juvenile HSPs or grandparent-grandoffspring pairs, which should be somewhat rarer than POPs on demographic grounds.

The number of POPs identified in this data set is 36. Four of the POPs found in 2017 using the DArTcap data are not in the 2018 set: this is due to changes in the QC checks which meant fewer fish were included in the comparisons this year. Including the POPs that were identified previously using microsatellites (recall that the genotyping method changed after 2015 from using microsatellites to DArTcap sequencing; see Bravington et al. 2015, 2017), we now have a total of 79 pairs[1]. The breakdown by juvenile birth year and adult capture year is given in Table 3.

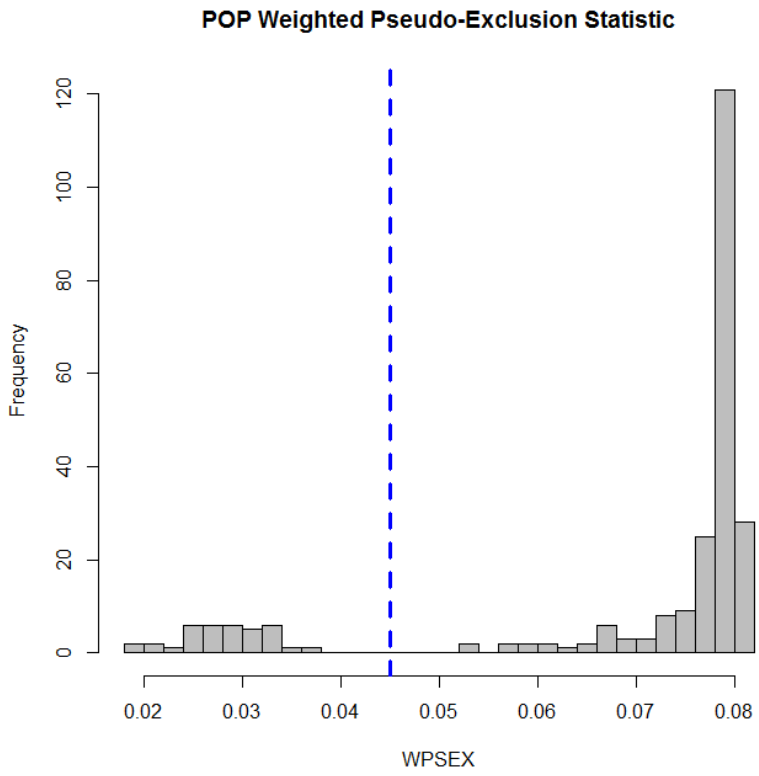


**Figure 2. Histogram of the weighted-pseudo-exclusion (WPSEX) statistic for identifying parent-offspring-pairs (POPs). Low values (below the vertical blue dashed line) indicate POPs. The x-axis is truncated at 0.08 to omit the gigantic peak of unrelated pairs to the right.**

**Table 3. Number of POPs (including those identified using microsatellites and DArTcap data) broken down by juvenile birth year (rows) and adult capture year (columns).**

| | 2006 | 2007 | 2008 | 2009 | 2010 | 2011 | 2012 | 2013 | 2014 | 2016 |
|---|---|---|---|---|---|---|---|---|---|---|
| **2002** | 0 | 0 | 0 | 0 | 0 | NA | NA | NA | NA | NA |
| **2003** | 0 | 5 | 1 | 2 | 0 | 0 | 0 | 1 | 0 | 1 |
| **2004** | 0 | 2 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 |
| **2005** | 1 | 4 | 5 | 4 | 1 | 0 | 0 | 1 | 2 | 0 |
| **2006** | NA | 4 | 3 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **2007** | NA | NA | 3 | 4 | 1 | 3 | 2 | 0 | 2 | 0 |
| **2008** | NA | NA | NA | NA | 0 | 1 | 1 | 1 | 0 | 0 |

[1] Note that 45 pairs were identified using microsatellites and 36 identified using DArTcap data, which gives a total of 81, but 2 of these are the same because the 2010 adult samples were run using both microsatellites and DArTcap and we are finding 2 POPs involving an adult captured in 2010 using both data sets.

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **2009** | NA | NA | NA | NA | 0 | 1 | 1 | 1 | 0 | 0 |
| **2010** | NA | NA | NA | NA | 0 | 0 | 1 | 4 | 0 | 2 |
| **2011** | NA | NA | NA | NA | 0 | 0 | 1 | 2 | 1 | 2 |
| **2012** | NA | NA | NA | NA | 0 | 0 | 0 | 1 | 1 | 0 |
| **2013** | NA | NA | NA | NA | 0 | 0 | 0 | 0 | 0 | 1 |

## 5.2 HSP-finding

HSPs were identified using the same method as in 2017, which uses a pseudo-log-odds-ratio (PLOD) statistic to measure the relative probability of a pair of fish having their observed genotypes if they are HSPs compared to if they are unrelated. The detailsare provided in Appendix C of Bravington et al. (2017).

Among 11,928 juveniles included in the HSP-finding analysis (i.e., 11928*11927/2 = ~71 million pairwise comparisons), we found 157 that we are confident are HSPs (and 4 that are full-sibling-pairs (FSPs)) based on the PLOD test statistic (Figure 3). The observed PLOD distributions for unrelated pairs and HSPs match the predictions of genetic theory (Figure 3, left), which gives us confidence in using this statistic to identify HSPs. Unlike the WPSEX statistic for identifying POPs, the PLOD statistic does not give a clear separation between the bump for HSPs and that (to the left) for unrelated/less-related fish; thus, we chose the lower cut-off value for HSPs to be 40 to ensure the number of false positives from unrelated/less-related pairs was minimal. An inevitable consequence of this means the true number of HSPs is expected to be about 14% higher than 157 because of false-negatives (calculated using the expected distribution for HSPs), but this is allowed for in modelling (Bravington et al. 2017). The division between PLOD values for HSPs and FSPs (to the right) was clear. The breakdown in numbers of identified HSPs by birth years is given in Table 4.

Note that the DArTcap data added to the analysis this year was not entirely consistent with the previous data (e.g., the sequence counts for some loci were significantly higher or lower on average than before). We are still investigating the cause of this difference and the implications might for kin finding in the future; there may need to be further modifications to the genotyping and/or analytical processes to improve consistency for future updates.

As a consequence, some modification to the genotype-calling process was required (which is being investigated further), and the separation in the PLOD ‘bumps’ for HSPs and unrelated/less-related fish is less clear this year, relative to the 2017 results (Bravington et al 2017). As a result, we needed to increase the lower PLOD cut-off value slightly, from 37 last year to 40 this year, in order to ensure false positive HSPs are rare. This means some pairs of fish that were deemed HSPs last year have been excluded from the set of HSPs this year. This difference is accounted for in the analysis, however, because the expected number of false negatives has increased accordingly from 10% last year to 14% this year.

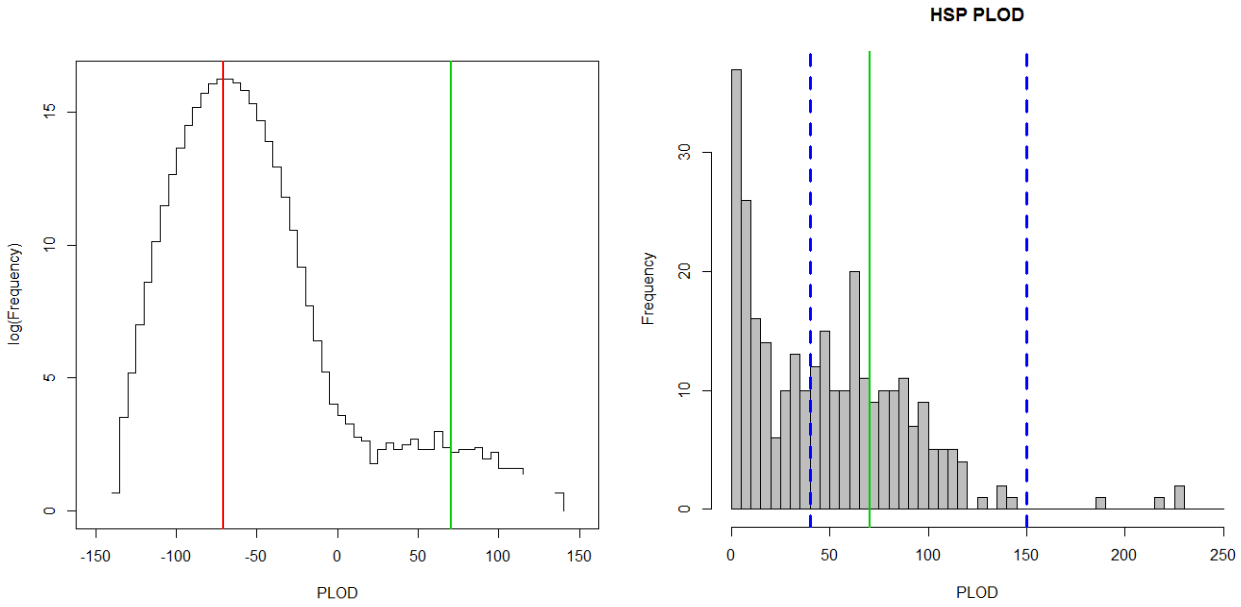


**Figure 3. (left) Log histogram showing the pseudo-log-odds-ratio (PLOD) statistic for every pairwise comparison of juvenile SBT (about 71 million comparisons). Red and green vertical lines are the theoretical means for unrelated pairs and half-sibling-pairs (HSPs) respectively. (right) Histogram of PLOD values that are above zero. Values between the two vertical blue dashed lines (chosen visually) indicate almost certain HSPs. Higher values (>150) indicate full-sibling-pairs (FSPs), and lower values (<40) indicate less-related and unrelated pairs, but will also contain some false-negative HSPs (see text).**

**Table 4. Number of HSPs broken down by birth year of younger sibling (rows) and older sibling (columns).**

| | 2003 | 2004 | 2005 | 2006 | 2007 | 2008 | 2009 | 2010 | 2011 | 2011 | 2012 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **2003** | 7 | 5 | 3 | 3 | 0 | 2 | 1 | 0 | 0 | 2 | 0 |
| **2004** | | 6 | 3 | 6 | 2 | 2 | 2 | 0 | 0 | 2 | 1 |
| **2005** | | | 4 | 3 | 3 | 3 | 0 | 5 | 1 | 1 | 0 |
| **2006** | | | | 10 | 5 | 1 | 3 | 7 | 4 | 0 | 1 |
| **2007** | | | | | 3 | 5 | 3 | 2 | 2 | 2 | 2 |
| **2008** | | | | | | 6 | 1 | 1 | 3 | 3 | 0 |
| **2009** | | | | | | | 1 | 2 | 0 | 0 | 0 |
| **2010** | | | | | | | | 3 | 1 | 2 | 1 |
| **2011** | | | | | | | | | 3 | 3 | 2 |
| **2012** | | | | | | | | | | 3 | 2 |
| **2013** | | | | | | | | | | | 3 |

# 6 Review of long term sample storage options

## 6.1 Tissue storage for DNA extraction

The reliability of polymerase chain reaction (PCR) for detecting genetic variation relies heavily on the ability to isolate DNA of acceptable quality and quantity. The amount of DNA isolated can be determined by the quality of tissue used, so adequate preservation of tissue samples is a prerequisite if future processing of tissue is required.

The preservation of tissue, and thereby the nucleic acids, relies on the inhibition of tissue nucleases and denaturation. There are several methods used to ensure the preservation of tissue including freezing, ethanol/EDTA, buffers and desiccation (Table 5).

Freezing is commonly used as a method of long term storage as low temperatures effectively inhibit enzymatic activity and generic DNA degradation. The price of -20°C and -80°C freezers are approximately AU$8,000 and AU$20,000 respectively (plus operating costs). However, DNA yields are greatly improved if buffers (e.g., EDTA) are also used and the samples are stored at -80℃. This is because the buffers partly protect against the degradation that occurs during temperature changes. CSIRO currently used this method of sample storage.

The use of ethanol has also proved to be a suitable method for tissue storage and the addition of EDTA buffer to 95% ethanol significantly increases DNA yield (Nagy 2010, Wasko et al. 2003, Dessauer et al. 1996). As the use of ethanol is relatively expensive, alternatives include EDTA buffers with added thymol and NaF. These buffers provide a relatively inexpensive storage media which yield high DNA and is also suitable for long term storage at -80℃. There is evidence that buffers containing dimethylsulfoxide (DMSO) provide a good-quality and cost-effective method for tissue preservation at this temperature as well. Only one method has been proven effective at room temperatures which involves using medium containing 20% DMSO, 0.25M sodium EDTA and NaCl to saturation (pH7.5), however optimal DNA yield was only tested for marine invertebrates (Haussermann 2004, Nagy 2010).

Freezing tissues in Liquid nitrogen (-195℃), although highly effective for long term preservation, is the most expensive method for tissue storage (Table 5).

Dry (desiccated) preservation is used for a wide variety of samples but it is generally not recommended for tissue samples. There is no evidence that the use of additives improves the storage condition of desiccated samples.

## 6.2 Extracted DNA storage

Replicate testing of stored DNA is often used to confirm results and to accommodate future testing with new technologies. The re-testing of stored DNA samples is reliant on ability to preserve DNA quality. Optimal storage of extracted DNA is achieved when DNA is maintained in a glassy state. In this state, molecules lose the ability to diffuse such that proton movement is greatly reduced and thereby prevents chemical and nuclease degradation.

Common storage methods include freezing (-20℃, -80℃ and -196℃), drying and storing at room temperature (Table 5). The glass state can be achieved by freezing. Storage of DNA at -20°C to -80°C can provide sufficient conditions depending on the quality and quantity of DNA desired and the time frame in which the sample will be stored; <1 year at -20°C, >1 year at -80°C (Lee et al. 2012, Roder et al. 2010). However, neither condition will maintain DNA quality equivalent to those stored at -196℃ over extended time periods (e.g. decades) (Lee et al. 2012, Baust 2008, Shewale and Liu 2013).

In contrast to freezing, it is possible to store DNA in a dry state. However, if moisture is added to the dry state, or the temperature is raised above the glass transition temperature of water, movement and reactivity is re-established and damage to DNA can occur (Yuanzheng and Angell

2005). A number of studies have shown that DNA samples stabilized in a synthetic storage medium are sufficiently protected from degradation during dry storage at room temperature (Lee et al. 2012, Smith et al. 2005, Bonnet et al. 2010). Samples stored in a storage medium have been reported to have between a 2 and 10-fold higher recovery of samples compared to equivalent samples stored in -80℃ conditions (Lee et al. 2012). There are two main storage mediums that have been tested with success; trehalose (Smith et al. 2005) and SampleMatrix™ (Lee et al. 2012). See Table 5 for relative cost estimates.

**Table 5. Common storage methods used for tissue and DNA preservation, adapted from Nagy (2010)**

| Sample | Storage type | Storage length | Effectiveness | Cost |
|---|---|---|---|---|
| **Tissue** | Freeze -20℃ | Short term | Only for short term storage | -20℃ freezer (~$8,000 AUD) and operating costs |
| | Freeze -80℃ | Long term | Effective | -80℃ freezer (~$20,000 AUD) and operating costs |
| | Freeze -80℃ & EDTA buffers | Long term | Effective (currently used at CSIRO) | -80℃ freezer (~$20,000 AUD), EDTA buffers and operating costs |
| | Freeze -80℃, EDTA buffers & Ethanol | Long term | Effective | -80℃ freezer (~$20,000 AUD), ethanol, EDTA buffers and operating costs |
| | Freeze in Liquid nitrogen (-195℃) | Extended periods | Highly effective | Very expensive |
| | Physical desiccation | Short term to long term | Not very suitable for aquatic specimens | Inexpensive |
| **Extracted DNA** | Freeze -20℃ | Short term (weeks) | Moderate | -20℃ freezer (~$8,000AUD) and operating costs |
| | Freeze -80℃ | Long term (years) | Effective, improved with buffers (currently used at CSIRO) | -80℃ freezer (~$20,000AUD) and operating costs. Plus buffers if used. |
| | Freeze in Liquid nitrogen (-195℃) | Extended periods (decades) | Highly effective | Very expensive |
| | Dried without storage medium | Long term (years) | Moderate | Specific equipment required. Time expensive |
| | Dried with storage medium | Long term (years) | Highly effective | Storage medium costs (brand dependant – generally expensive), Specific equipment required. Time expensive |

## 6.3 Annual storage requirements

After processing is complete, SBT muscle tissue and extracted DNA samples for close-kin mark recapture (and gene tagging) are stored at CSIRO in freezers at -80°C. This allows for future DNA extraction of tissue if required or replicate testing of previously extracted DNA, and appears to be the logical option for long term storage of samples.

A typical 950 litre -80°C (ultra-low temperature) freezer can store aproximately 60,000 muscle tissue samples in 100 well freezer boxes or 130,000 extracted DNA samples in archive plates. Based on the current CKMR and GT projects, ~22% of a freezer is required to store samples each year, thus a freezer will reached capacity every 4.5 years (Table 5).

If CKMR (and gene tagging) projects are ongoing, long term storage needs for tissue and extracted DNA samples must to be considered, given that ultra low temperature freezers (and space to house freezers) is finite at CSIRO. As far as we are aware, there are no commercial facilities that can store samples at -80°C in Hobart. Investigation of alternatives storage solutions and maximum retention time for samples are recommended.

**Table 5. Long-term storage needs for close-kin mark recapture (CKMR) and gene tagging (GT) project.**

| Project | Tissue | Extracted DNA | Total |
|---|---|---|---|
| **CKMR – samples/yr** | 3100 | 2024 | |
| **GT – samples/yr** | 0 | 20,000 | |
| **Total** | 3100 | 22040 | |
| **Freezer capacity** | 60,000 | 130,000 | |
| **% of freezer needed/yr** | 5% | 17% | 22% |
| **Capacity reached (yrs)** | | | 4.5 |

# 7 Summary

The project successfully completed:

1) 2017/18 tissue sampling in Australia and Indonesia (juveniles aand adults);
2) 2016/17 tissue subsampling and DNA extraction. DArT will complete the genotyping before the end of the project.
3) 2015/16 kin finding (POPs and HSPs).

An updated dataset of identified SBT parent-offspring pairs and half-sibling was provided to the CCSBT in April 2018, in the correct format for the operating model code. Note, however, that the 2015/16 DArTcap data added to the analysis this year was not entirely consistent with the previous sequencing data (e.g., the sequence counts for some loci were significantly higher or lower on average than before). We are still investigating the cause of this difference and the implications might for kin finding in the future; there may need to be further modifications to the genotyping and/or analytical processes to improve consistency for future updates.

Assuming, the CKMR (and gene tagging) projects remain part of the ongoing monitoring of the stock, long term storage needs for archived tissue and DNA samples must to be considered. Ultra low temperature freezer space (and space to house freezers) at CSIRO is a finite resource, and as far as we are aware, there are no commercial facilities that store samples at -80℃ in Hobart. Investigation of alternatives storage solutions and maximum retention time for samples are recommended.

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