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DRAFT Final Report to TRAFFIC International

Genetic species identification – SBT market presence in China.

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Report to TRAFFIC and CCSBT Secretariat

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1 Introduction

CSIRO has developed and refined a specific DNA assay for identification of individual *Thunnus* species and skipjack tuna for widespread commercial application (e.g. Chain of Custody for market certification and Catch Documentation Schemes for tuna RFMOs) as part of a strategic research program on Next Generation Sequencing methods for fisheries monitoring assessment and management (Davies and Grewe 2015; Davies at al 2015; Grewe et al., 2016). The assay will clearly and reliably distinguish among the primary sashimi-grade species of tuna (southern bluefin tuna, Atlantic bluefin tuna, Pacific/Northern bluefin tuna, bigeye tuna and yellowfin tuna) as well as albacore and skipjack.

CSIRO was approached by TRAFFIC to provide specific specific identifications of tissue samples collected from a range of restaurants in Shanghai and Beijing, China, as part of a larger review and trade study on the presence of SBT in Chainese seafood markets commissioned by CCSBT (Anon., 2015). This report summarises the the work undertaken and the results of species identification of the samples provided by TRAFFIC.

2 Objectives

1. Extract the DNA from 100 tissue samples using standardised protocols and profile for species identification.
2. Validate the species ID test for a subset of samples using the Genotyping-by-Sequencing approach (ddRAD).

3 Methods

Sampling collection

A total of 200 muscle tissue samples from sashimi-grade tunas were purchased from sushi restaurants by TRAFFIC. Sampling was stratified across cities, months and price category of restaurants (Table 1) in an attempt to provide as representative sample of the product being sold as sashimi grade tuna in the same retail markets as an earlier 2012 study. The samples were all collected from restaurants in Beijing and Shanghai, preserved in RNA-Later following standard protocols and transported to CSIRO.

DNA extraction

DNA was extracted from a 10mg sub-sample of tissue from 199 samples. A bead-based extraction protocol (Machery Nagel Nucleomag) kit was used on an Eppendorf EP motion robot to produce a 150uL archive solution and 50uL working stock of DNA in micro-titre format plates. Archive plates of extracted DNA are stored in dedicated -80^{o}C freezers at CSIRO Hobart. The working stock plates of extracted DNA were shipped to Diversity Arrays Technology (DArT) in Canberra for genotype sequencing of approximately 5000 single nucleotide polymorphism (SNP) loci.

Table 1. Summary of sampling design for estimating proportion of tuna species occurring in sushi restaurants in Beijing and Shanghai in two rounds of sampling in January and March 2016. One sashimi sample was purchased from each restaurant in each price class sampled in each month. Price classes were: 0-100, 101-200, 201-300, 301-400, 400-500, >500.

CITY	MONTH	PRICE CLASS	# RESTAURANTS
Beijing	Jan	5	10
	Mar	5	10
Shanghai	Jan	5	10
	Mar	5	10
		Total	**200**

DNA Profile Analysis

DNA profiles consisted of information collected from 5000 SNP loci for each individual. Genetic distances (i.e. percent difference between two individuals) were calculated for pairwise comparisons of all TRAFFIC samples as well as comparisons to DNA profiles from control samples of known 8 *Thunnus* and Skipjack tuna species previously genotyped by CSIRO. Sequencing artefact errors on the Illumina sequencer occur at 1% or less frequency and thus individuals with less than 1% differences were considered to be samples of the same individual. Percent sequence differences of 2-3% were considered conspecifics and assigned the identification of the matching control species.

Estimation of Proportion of SBT in Sushi Product from Restaurant Survey

Of the 200 samples collected, one from Beijing samples was not genotyped, and one of the restaurants did not have a price category recorded, leaving 198 samples for this analysis (Table 2).

Table 2. Summary of tuna samples used in analysis of the proportion of SBT in sushi sold in Beijing and Shanghai.

	Beijing		Shanghai	
Price category (CNY)	January	March	January	March
<100	1	6	0	8
100-200	9	28	11	24
200-300	10	13	9	10
300-400	11	1	12	4
400-500	9	1	8	0
>500	10	0	10	3
Total	**50**	**49**	**50**	**49**

The stratified rangom survey design provided the basis to estimate the proportion of SBT in the sushi restaurant market in the two cities using the number of restaurants in each class and the proportion of SBT in each class (Table 2). A binomial generalized linear model (GLM) was fit to the proportion of tuna samples identified as SBT with the *city*, *month* and *price* category of the restaurant as explanatory variables (The glm function in R with family set to binomial with a logit link was used. See Appendix A). *Month* was not significant so was dropped from the final model.

The final model was used to predict the proportion of SBT in each city and restaurant price category (Table 2). Then, using the number of restaurants in each city belonging to each price category, taken from Table 6 of the TRAFFIC interim report to CCSBT (CCSBT-ESC/1609/36), a weighted average of the predicted SBT proportions was calculated to get the expected proportion of tuna samples that are SBT (i) across both cities and (ii) in Shanghai alone (since only 1 of the Beijing samples was SBT). The weighted average across both cities is 0.165, and the weighted average for just Shanghai is 0.243.

Table 2. Predicted proportion of samples that are SBT by city and restaurant price category.

City	Price Category	No. samples	No. SBT	Observed proportion SBT	Predicted proportion SBT	No. restaurants	Weight
Beijing	<100	7	0	0	0.009	435	0.160
Shanghai	<100	8	2	0.250	0.242	1169	0.431
Beijing	100-200	37	0	0	0.006	300	0.111
Shanghai	100-200	35	6	0.171	0.165	375	0.138
Beijing	200-300	23	0	0	0.019	105	0.039
Shanghai	200-300	19	8	0.421	0.398	150	0.055
Beijing	300-400	12	1	0.083	0.021	33	0.012
Shanghai	300-400	16	6	0.375	0.422	75	0.028
Beijing	400-500	10	0	0	0	10	0.004
Shanghai	400-500	8	0	0	0	17	0.006
Beijing	>500	10	0	0	0.005	21	0.008
Shanghai	>500	13	2	0.154	0.150	21	0.008

4 Results and Discussion

A total of 199 individual DNA profiles were obtained using 5000 SNP loci. One sample was ommmitted from the genotyping runs as it was not possible to fit all 200 restaurant samples and the necessary control samples in the allocated plates.

Based on percent differences in SNP sequence, individuals were unambiguously classified as belonging to skipjack or one of eight *Thunnus* species (Table 3). Members of identified species groups had percent sequence differences that ranged between 2-3% while species mis-matches had observed percent sequence differences greater than 5%. This clear range of percent differences and the use of verified controls ensured very high probability of correct assignment to species.

Bigeye tuna was the most frequent species found at 35% of the 199 fish analysed. The three bluefin species were present at lower frequencies: Pacific bluefin (28%), Atlantic bluefin (17%) and southern bluefin (13%).

Southern bluefin samples were almost all sampled from restaurants in Shanghia (Table 4). Only one southern bluefin was sampled in Beijing. And, interestingly, the large majority of southern bluefin samples were in the lowest price class, 0-100 (Table 5), with the small number of samples in high prices classes being for belly meat.

Yellowfin was the least common species present at only 7% of the toal sample, while the remaining species (skipjack, albacore, blackfin, and longtail tuna) were not present in the tissues sampled.

Indicative of the power of the genotyping method, tissue from 6 individual fish were identified as having been sampled at more than one restaurant (Table 6). This result may not not be surprising if the duplicate samples were from a single large fish which had been processed into the various sushi cuts and distributed among restaurants.

Table 3. Summary of species identification results from 199 tissue samples from sushi restaurants in Beijing and Shanghai, China. ABT = Atlantic bluefin tuna, PBT = Pacific bluefin tuna, BET = bigeye tuna, SBT = southern bluefin tuna, YFT = yellowfin tuna, ALB = albacore tuna, BLK = blackfin tuna, LOT = longtail tuna and SKJ = skipjack tuna.

SPECIES ID	COUNT	PROPORTION
BET	70	0.35
PBT	55	0.28
ABT	34	0.17
SBT	26	0.13
YFT	14	0.07
ALB	0	0.00
BLK	0	0.00
LOT	0	0.00
SKJ	0	0.00
Total	**199**	**1.00**

Table 4. Summary of the number of southern bluefin tuna identified in samples collected by city and sampling month.

CITY	JANUARY	MARCH	TOTAL
Shanghai	10	15	25
Beijing	1	0	1
		Total	**26**

Table 5. Summary of the number of southern bluefin tuna identified in samples by price class.

PRICE CLASS	COUNT
0-100	21
100-200	4
200-300	1
300-400	0
400-500	0
>500	0
Total	**26**

Table 5. Summary of the duplicate tuna identified by genetic profiles in samples collected by city and sampling month (n= 6 duplicates).

City	Shanghai	Shanghai	Beijing	Beijing	Beijing	Beijing	Beijing	Beijing	Beijing	Beijing	Beijing	Beijing	Beijing	Beijing
Source	Canada	Canada	Kagoshima, Japan	Japan	Indonesia	Japan	Japan	Spain, from agent	Nagasaki, Japan	Atlantic	Nagasaki, Japan	Japan/Canada	Japan	Spain
Shown on menu	Bluefin tuna	Toro sashimi	Tuna Sashimi	Bluefin tuna	Fresh Bluefin Tuna	Tuna	Bluefin Tuna	Tuna	Tuna Sashimi	Bluefin Tuna Sashimi	Bluefin tuna	Bluefin Tuna Sashimi	Tuna Sashimi	Bluefin Tuna Sashimi
Date sampled	7 Jan	9 Jan	19 Jan	15 Jan	18 Jan	15 Jan	19 Jan	15 Jan	25 Jan	9 Mar	16 Mar	14 Mar	15 Mar	15 Mar
Species ID	ABT	ABT	NBT	ABT	NBT	NBT	NBT	NBT	NBT	ABT	ABT	ABT	ABT	ABT
Sample number	21	38	53	58	74	93	94	95	100	167	173	183	194	195
21	Duofan Manyi Jingshi	SOMA LOUNGE												
53			Fukubuku Sake Bar & Lounge (Chaoyang)		Haiku Japanese Restaurant (Xicheng)	Yotsuba Sushi (Chaoyang)			Nobu Beijing (Chaoyang)					
58				Kenzan Sushi (Chaoyang)										IZAKAYA (Jianguolu
94							Ran Sushi (Chaoyang)	Tianshe Teppanyaki (Chaoyang)						
167										Shotamuni Restaurant (Xinshijie)		Shotamuni Restaurant (Orient Plaza)		
173											AKIMOTO		Akimoto (2nd)	

Key

Colour code	Explanation
	1st observation of individual fish
	1st observation of duplicate individual
	2nd observation of duplicate individual
	3rd observation of duplicate individual

5 Summary

The results demonstrate that the ddRAD method used here can clearly and unambiguously identify individual tuna species, including southern blufin tuna with a high degree of accuracy and consistency. Furthermore, it was possible to identify individual fish that were sampled multiple times among different restaurants during the realtively short period of the study, further demonstrating the power of these methods for identifying and or tracking product through the supply chain.

In terms of southern bluefin tuna, the results demonstrate:

- SBT is being sold in retail sushi restaurants in China, representing ~16.5% of sales in the two major cities during the short period of this study .
- SBT appears to be much more common in the Shanghai market and, predominantly, in the lower price class of restaurant. Although there we examples of it being sold in mid-higher tier classes of restaurant. For the period of the study it constituted ~24.3% of the sashimi grade tuna sold through sushi restaurants in Shanghai.
- The product is generally not being sold as southern bluefin tuna. The most common retail name being “Bluefin tuna” and “Tuna Sushimi”.

Naturally, the somewhat restricted scope of the sampling (the two major cities and two months in the first quarter of 2016) and the strong difference in the presence of SBT between Shanghai (26 observations) and Beijing (one occurrence) means some care needs to be taken in extrapolating these results to the presence of SBT in the wider Chinese market. However, these results and the ancillary data collected by TRAFFIC, as part of the sampling protocols for the study, indicate that SBT represents a substantial proportion of the sashimi market in Shanghai (at least), that it is rare for it to be transparently identified as SBT in the retail market, and there are a range of stated sources of the product being sold in restaurants.

6 References

Davies, C.R. and Grewe, P.M. 2016. Development of genetic markers for identification of skipjack tuna from the Maldivian Islands and standardised protocols for their use. *Draft final Report to the Marine Stewardship Council*. January 2016.

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Grewe, P.M., H. E. Irianto, C. H. Proctor, M. S. Adam, A. R. Jauhary, K. Schafer, D. Itano, K. Evans, A. Killian and C. R. Davies. 2016. Population structure and provenance of tropical tunas: recent results from high throughput genotyping and potential implications for monitoring and assessment. *Working Paper SA-WP-01 to the Scientific Committee of the Western Central Pacific Scientific Committee*, Bali, Indonesia, August 2016.

Attachement A: The R output for the final GLM.

```
Call:
glm(formula = Species == "SBT" ~ City + PriceClass, family = binomial(link
= "logit"), data = china.dat)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-1.0472  -0.6014  -0.1942  -0.1006   2.7873

Coefficients:
                      Estimate Std. Error z value Pr(>|z|)
(Intercept)            -4.6910     1.2845  -3.652 0.000260 ***
CityShanghai            3.5495     1.0412   3.409 0.000652 ***
RestClass(100,200]     -0.4768     0.9229  -0.517 0.605407
RestClass(200,300]      0.7295     0.9254   0.788 0.430473
RestClass(300,400]      0.8273     0.9460   0.875 0.381835
RestClass(400,500]    -16.4081  1362.9845  -0.012 0.990395
RestClass(500,2e+03]   -0.5934     1.1132  -0.533 0.594006
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

(Dispersion parameter for binomial family taken to be 1)


    Null deviance: 150.17  on 197  degrees of freedom
Residual deviance: 109.21  on 191  degrees of freedom
  (1 observation deleted due to missingness)
AIC: 123.21

Number of Fisher Scoring iterations: 17

Analysis of Deviance Table
          Df Deviance Resid. Df Resid. Dev  Pr(>Chi)
NULL                        197     150.17
City       1   29.327       196     120.84 6.114e-08 ***
PriceClass 5   11.637       191     109.21   0.04012 *
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
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