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PCR-based sex determination for North Pacific Albacore (*Thunnus alalunga*)

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Abstract

Albacore (*Thunnus alalunga*) is a pelagic tuna species that supports a lucrative fishery worldwide. Like all tuna species, Albacore are not sexually dimorphic. This means that accurate identification of sex in Albacore is only possible through direct observation of gonads. This process is costly, time consuming, and lethal, often necessitating histological confirmation of sex due to the large numbers of immature animals captured in some fisheries. Previous work has shown that a genetic approach to determine sex in Albacore is possible. To explore this, we examined sequence data in Albacore and modified previous genetic assays in an attempt to decrease phenotype/genotype mismatches in a PCR-based method. The modified assay presented herein, when combined with previous data, demonstrates that sex of albacore can be determined with a 3.3% error rate, and that increased sample size will help to refine this.

Revision Note January 21, 2021

This revision reflects an error in Table 1 of the original document. The original table incorrectly listed primer sca64_3726411_R_T (CTGATGTCCTCTGTAACACAATCAT). The correct primer is sca64_3726411_R_A (CTGATGACCTCTGTAACACAATCAT). This revision also corrects the PCR recipe from 0.5 units of TAQ to 0.25 units TAQ. Changes are in **bold** and highlighted in **grey**.

Introduction

Albacore (*Thunnus alalunga*) is a pelagic tuna species that supports a lucrative fishery worldwide. Currently, Albacore in the Pacific is managed as two separate stocks, one in the North Pacific Ocean and one in the South Pacific Ocean. Albacore are highly migratory and, as such, management requires an international effort. The Albacore Working Group (ALBWG) of the International Scientific Committee for Tuna and Tuna-like Species in the North Pacific Ocean (ISC) is tasked with conducting regular stock assessments of north Pacific Albacore to estimate population parameters, summarize stock status, and develop scientific advice on conservation needs for fisheries managers. These assessments are provided to various international governments and Regional Fishery Management Councils. The most recent stock assessment for Albacore was completed in 2017 (ALBWG 2017).

The 2017 Albacore stock assessment implemented several improvements over the previous assessment (2014). Among them was the use of age and sex-specific natural mortality parameters, rather than a single parameter (Kinney and Teo, 2016; ALBWG,

2017). In Albacore, data suggest that sex ratio is dynamic and may change within a cohort over time (ALBWG, 2017). It is estimated that sex ratio is approximately 1:1 until albacore reach age 3, after which males become more common due to a higher estimated mortality in females beyond this age (2017 ISC assessment). This is further accentuated by potential differences in growth, and the sex ratio is heavily male biased for albacore >100 cm (Farley et al. 2013). Whether these phenomena are related to selectivity of the fishery or reflective of natural processes is unclear. Among the key uncertainties expressed in the 2017 stock assessment was the lack of sex-specific size/age data.

Like all tuna species, Albacore are not sexually dimorphic. This means that accurate identification of sex in Albacore is only possible through direct observation of gonads. This process is costly, time consuming, and lethal, often necessitating histological confirmation of sex due to the large numbers of immature animals captured in some fisheries. As a result, the sex ratio and sex-specific size information for Albacore fisheries which operate throughout the North Pacific Ocean are uncertain. Given the importance of sex in the determination of mortality rate, as well as the identified key uncertainty of sex-specific size data in the 2017 assessment, tools that can improve the efficiency of determining the sex of an individual are highly desirable.

Over the past decade, and with the advantages of high-throughput, next-generation DNA sequencing technology, genetic methods for the determination of sex in fishes have been developed for several fishes. In 2019, Suda et al. published an improved genome for the Pacific Bluefin Tuna (PBF; *Thunnus orientalis*) and identified regions of the genome that contained multiple single nucleotide polymorphisms (SNPs) that corresponded with the sex of the individual. Suda et al. (2019) developed three simple PCR assays that were able to identify male PBF individuals based on these sex-linked SNPs.

Given the close relationship and genetic similarity between PBF and Albacore, the potential for using the PBF sex markers of Suda et al. (2019) exists. Chiba et al. (2019) tested the efficacy of these assays in Albacore from port samples in Japan, finding good concordance between phenotype and genetic sex with some discrepancy (n=105; 94.3%, 100%, and 97.1% for Suda et al.'s 2019 Pair I, II, and III, respectively). We further tested the Suda et al. (2019) primer sets in additional albacore samples from the northwest Pacific fishery off Oregon and Washing, USA, (n=16), however found phenotype/genotype mismatches in all three markers presented by Suda et al. (2019). We hypothesized both that there may be Albacore-specific heterozygosity at some of the primer sites and that use of a mitochondrial gene as a control marker (see Methods) may increase false positive female error because a primer mismatch in the male specific primers could cause amplification failure in a genotypic male, but may not prevent the mitochondrial gene from amplifying. In addition, mitochondrial genes are often easier to

amplify than nuclear genes even with sub-optimal DNA samples. To explore this, we examined sequence data from the genomic region that contains Suda et al.'s (2019) sex markers in Albacore and explored potential modifications to the Suda et al. (2019) assays in an attempt to decrease phenotype/genotype mismatches.

Methods

Initially, the Polymerase Chain Reaction (PCR) was used to genetically determine sex in Albacore following the methods of Suda et al. (2019) for Albacore. This protocol was a multiplex reaction that included both male-specific sex marker primers and a control primer set that amplified a short region of the mitochondrial ND4 gene. This control allowed for a visual indication that the PCR reaction had not failed if the male marker did not amplify, thus verifying that the sample displayed the female pattern (control band only). Similar to the results of Chiba et al. (2019), we found instances of both false positive females (phenotypic males with female genotypes) and false positive males (phenotypic females with male genotypes) when using Suda et al's (2019) male-specific primer pairs. In combination with the data presented by Chiba et al. (2019), Suda et al.'s (2019) "Pair II" had the least overall mismatch rate, thus we explored a modification to their assay using this marker.

In order to examine the homologous region in Albacore that contained the sex markers of Suda et al. (2019), we first examined the portion of PBF scaffold 64 that contained the sex-linked SNP markers. We designed primers flanking Suda et al.'s (2019) priming sites and applied them to Albacore (primer sequences and methods available upon request).

In Albacore, we verified a contiguous two base pair heterozygosity in males corresponding to the 3' end of Suda et al.'s (2019) "Pair II" male-specific reverse primers (sca64_3726411_R_A and sca64_3726411_R_T). We also noted that Suda et al.'s (2019) sca64_3726411_R_A had no corresponding sequence in Albacore, thus we only used sca64_3726411_R_T in subsequent analysis.

To address the potential for error due to the use of the mitochondrial control marker we designed additional Albacore specific primers such that a three primer cocktail could be used to create an assay targeted on the same genomic region that would produce an ~400 bp fragment in both males and females, and an additional ~250 bp fragment only in males (Table 1; Figure 1). Given that the 3' end of the male-specific primers for the shorter fragment incorporated the two base pair heterozygosity on its 3' terminus, mis-priming on genotypic females had a low probability of extension (Simsek and Adnan, 2000) thus also reducing the chances of false positive males.

For the new three-primer assay, 10 µL PCR reactions were prepared with 0.2mM each dNTP, 0.25µM of each primer, 0.5 mg/mL Bovine Serum Albumin (BSA), and **0.25U** standard *Taq* polymerase and buffer (New England Biolabs, Ipswitch, MA, USA). Following an initial denaturing step at 94°C for 3 min, 40 cycles of the following thermal cycling were performed: 94°C for 30s, 62°C for 30s, and 72°C for 30s. A final extension at 72°C for three min was performed. PCR products were visualized on a 2% agarose gel stained with ethidium bromide.

Results

Eighty two Albacore (33 male and 49 female) for which sex had been determined histologically were analyzed using the three primer PCR assay described above. Of the 33 histologically confirmed (phenotypic) males, only one (3.0%) presented with a female genotype (Table 2). Of the 49 histologically confirmed (phenotypic) females, five (10.2%) presented with a male genotype (Table 2). Overall, six of the 82 individuals (7.3%) presented with a genotype that differed from their histologically confirmed (phenotypic) sex (Table 2).

We successfully obtained sequence data for 4 of the 6 female/male phenotype/genotype mismatches. In all instances, the apparently male-specific priming region was an exact match to the primer as expected in phenotypic males. Unfortunately the sequencing for the single male/female phenotype/genotype failed upon first trial and due to present circumstances we have been unable to obtain sequence data for it or the remaining female/male phenotype/genotype mismatch.

Discussion

Sex ratio is an important component when evaluating population dynamics, especially if unequal contributions by males and females to reproduction are suspected. Given the fact that Albacore are not sexually dimorphic, and that there is an indication that sex ratio of a cohort may change with time, it is important to develop alternative, non-invasive, and cost-effective methods to determine the sex of an individual. Similar to the results of Chiba et al. (2019), we found that primer set “Pair II” of Suda et al. (2019) showed the most promising results for use in Albacore.

This modified assay for the “Pair II” marker shows that the sex of an individual Albacore can be determined with an overall error rate of 7.3% (n = 82) with a likely reduction in false positive female error. The error rate in our data is skewed, however, for males and females, with a smaller genotype/phenotype mismatch in males (3.0% vs. 10.2%, n = 32

and 34, respectively). When combined with the data from Chiba et al. (2019), the error rate for males is 1.2% (n = 79), for females is 4.8% (n = 108), and overall is 3.3% (n = 187). While these data may not be directly comparable for females, they likely provide a closer estimation of the true error rate of this assay. Further increases in sample size using the modified assay will help to refine error rates in both sexes.

It is important to remember that this assay only examines a small region of the Albacore genome that appears to be associated with sex. This genetic "marker" should not be presumed to be a causal factor in the phenotypic display of male or female characters which is almost certainly determined by a host of genetic, epigenetic, and environmental factors during development.

There are four main reasons for which a genotype/phenotype mismatch may occur: 1. The individual may be incorrectly identified as a male or female histologically, 2. Cross contamination at any point in the sampling/preserving/analysis stage, 3. The two base pair mutation being tested in males is not perfectly associated with sex, and/or 4. There is a portion of the population that fails to differentiate and develop to their genetically determined sex. We are unable to rule out cross contamination, however we are confident that, if present, it is at a very low level.

For the 82 samples examined in the present study, 26 of the original histological slides were unavailable to confirm the initial determination, however each slide was examined by two independent researchers and we are confident in the correct assignment. Among these, only two showed a genotype/phenotype mismatch (one M/F and one F/M genotype/phenotype mismatch). For the remaining 56 samples for which we were able to re-examine the histological preparations, 4 presented with a genotype/phenotype mismatch (all M/F genotype/phenotype mismatch). None of the original histological determinations in these 56 samples was found to be incorrect. Sequence data of some of the mismatched individuals (phenotypic female, genetic male) showed that these individuals contained the two assayed heterozygous SNPs and multiple other sex associated SNPs, indicating the assay performed as designed but there is imperfect match between genotype and phenotype. This strongly suggests that in Albacore, and for this assay, that there is a small portion of the population that presents with a genotype/phenotype mismatch.

While Chiba et al. (2019) showed 100% phenotype/genotype match using primer set "Pair II", it is difficult to rule out false positive females given the use of a mitochondrial gene as a "control" for reaction success. This modified assay amplifies the same region of the genome for males and females, thus reducing the possibility of false positive females. This assay also allows for phenotype/genotype mismatches to be further explored by

sequencing. It is anticipated that the genotype/phenotype mismatch rate will be refined as more samples with histologically confirmed sex are made available and analyzed.

References

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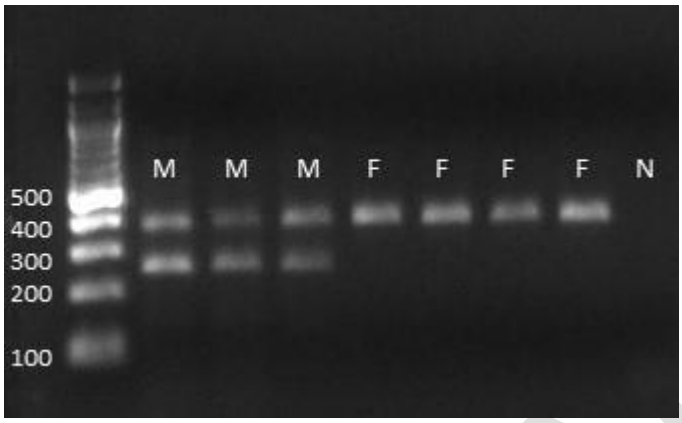

Figure 1. PCR bands showing male and female pattern for three-primer PCR sex determination assay described herein. M = male, F = female, N = no-template negative control. Numbers indicate fragment size of reference bands in base pairs.

Table 1. Primer set used in PCR analysis of sex in Albacore. **This table reflects a change to original document.**

Primer Name	Primer Sequence (5' - 3')	Source
sca64_3726411_1af	CAACAACTTGGAGCTTGCTG	This Study
sca64_3726411_R_A	**CTGATGACCTCTGTAACACAATCAT**	**Suda et al., 2019**
Set_2_R_Univ	TCAGGAAGTGTTGGGTCAC	This Study

Table 2. Summary statistics for matches/mismatches of phenotypes and genotypes for Albacore using the three-primer PCR assay described herein.

Category	N	Match/Mismatch	%Mismatch
Male	33	32/1	3.0
Female	49	44/5	10.2
Overall	82	76/6	7.3