Vol. 659: 175–184, 2021
https://doi.org/10.3354/meps13563

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published February 4

Effectiveness and potential application of sex-identification DNA markers in tunas

Satoru N. Chiba[1,5,*], Shinpei Ohashi[1], Fumiya Tanaka[1], Ayako Suda[2], Atushi Fujiwara[2], Derke Snodgrass[3], Hidetada Kiyofuji[1], Keisuke Satoh[1], Nobuaki Suzuki[1,4]

[1]National Research Institute of Far Seas Fisheries, Japan Fisheries Research and Education Agency, 5-7-1 Orido, Shimizu, Shizuoka 424-8633, Japan

[2]Research Center for Bioinformatics and Biosciences, National Research Institute of Fisheries Science, Japan Fisheries Research and Education Agency, Yokohama 236-8648, Japan

[3]Sustainable Fisheries Division, NOAA Fisheries, 75 Virginia Beach Drive, Miami, FL 33149, USA

[4]Fisheries Agency, Ministry of Agriculture, Forestry and Fisheries, 1-2-1 Kasumigaseki, Chiyoda, Tokyo 100-8907, Japan

[5]*Present address:* Hokkaido National Fisheries Research Institute, Japan Fisheries Research and Education Agency, 116 Katsurakoi, Kushiro, Hokkaido 085-0802, Japan

ABSTRACT: Sex-identification DNA markers are useful tools for sexing organisms that lack externally visible sexual dimorphism, and thus, they provide biological information for ecological and evolutionary studies. Tunas of the genus *Thunnus* (Scombridae), which comprises 8 species, lack sexual dimorphism of external morphology or coloration. In this study, we applied recently developed genotypic sex-identification markers for Pacific bluefin tuna to other tuna species to evaluate their effectiveness in sex identification. A sex-identification marker named 'primer pair II' demonstrated relatively high effectiveness in all tuna species, except southern bluefin tuna. Primer pair II was further tested in 209 albacore individuals collected during the scientific observer program onboard Japanese commercial long-line vessels, and it demonstrated robust performance for genotypic sex identification. The sex ratio of this albacore sample (1:1.4) significantly deviated from the expected 1:1 with the dominance of males, and the mean body size of males was higher than that of females. As all cross-species amplifications of the male-specific markers, except those for the southern bluefin tuna, were male-heterozygous polymorphisms, it is likely that a male-heterozygous sex-associated region exists in the *Thunnus* genome. The evolution of sex-determination systems in tunas was analyzed by ancestral state reconstruction, which showed that a common ancestor, before the evolution of the genus, possessed the male-heterozygous sex-associated genome region.

KEY WORDS: Sex-specific PCR · *Thunnus* · Male heterogametic sex · Sex ratio · Sexual dimorphism · Sex determination

1. INTRODUCTION

Sex-identification DNA markers are useful tools for sexing organisms that lack sexual dimorphism of external morphology and/or coloration, and thus, they provide biological information for ecological and evolutionary studies. Gonochoristic vertebrates have 2 major types of sex-determination (SD) systems: (1) genotypic SD (GSD), in which the sex is determined at conception, and genetic differences between the sexes are expected; and (2) environmental SD (ESD), in which sex is determined after fertilization according

*Corresponding author: chiba_s@affrc.go.jp

to environmental conditions, and there are no consistent genetic differences between the sexes (Penman & Piferrer 2008). Teleosts display diversity in the SD systems, ranging from ESD to strict GSD, such as the XX/XY, XX/XO, ZW/ZZ, ZW/ZO, X1X1X2X2/X1X2Y, XX/X1X2Y, and W1W2Z/ZZ systems (e.g. Schartl 2004, Penman & Piferrer 2008), and different GSD systems have evolved in some closely related species (Takehana et al. 2007).

The genus *Thunnus* comprises 8 species: Pacific bluefin *T. orientalis* (PBF), Atlantic bluefin *T. thynnus* (BFT), southern bluefin *T. maccoyii* (SBF), albacore *T. alalunga* (ALB), bigeye *T. obesus* (BET), yellowfin *T. albacares* (YFT), longtail *T. tonggol* (LOT), and blackfin *T. atlanticus* (BLF). Pacific bluefin tuna has the XX/XY SD system with male-heterozygous polymorphisms (Agawa et al. 2015), but the SD systems in other tuna species are unknown. Sexual size dimorphism, wherein adult males attain a larger size than females, occurs in several tuna species, including PBF (Shimose et al. 2016), BFT (Santamaria et al. 2009), SBF (Lin & Tzeng 2010), ALB (Chen et al. 2012), BET (Farley et al. 2006), and YFT (Shih et al. 2014). However, the sexual differences in growth and the SD system remain unknown owing to uncertainties in the sex identification of juveniles and/or tuna products. Improved understanding of both SD systems and sexual dimorphisms will help implement reliable stock management and/or efficient aquaculture, considering the evolution of sex and the ecological traits in tunas.

Tunas lack sexual dimorphism in external morphology and coloration, which makes visual sex identification difficult. The sex of tunas has been identified by inspecting gonad morphology (e.g. Williams et al. 2012, Ashida et al. 2015, Okochi et al. 2016), but in immature gonad stages, sex identification requires a high skill level and is laborious and time-consuming. Recently, highly accurate genotypic sex-identification assays using DNA markers have been developed for PBF (Suda et al. 2019). Suda et al. (2019) isolated 250 male-specific, male-heterozygous single nucleotide polymorphisms (SNPs) in PBF, from more than 30 million polymorphisms identified using genomic re-sequence data of 15 males and 16 females. They reported 3 primer pairs for sex-identification markers for PBF. Primer pairs I and II produced male-specific amplicons of 113 and 143 bp, respectively. Primer pair III produced a 142 bp male-specific amplicon and a 149 bp consensus amplicon from both sexes. If these primer pairs can enable the sex-identification of other tuna species, our understanding of sexual dimorphisms and SD system evolution in tunas can be improved.

The aims of this study were to (1) apply the recently developed PBF genotypic sex-identification markers to other *Thunnus* species and evaluate their effectiveness for sex identification, and (2) assess the potential of these sex-identification markers for practical implementation in ALB. In addition, an evolutionary hypothesis of the SD system in tunas was generated by analyzing the phylogenetic pattern of the male-heterozygous polymorphisms using an ancestral state reconstruction (ASR) method. The results showed that the markers are effective for all tested tuna species, except SBF, and that they could be employed for the sex identification of tuna products and juveniles in wild populations to advance our understanding of sex ratio and sexual dimorphism.

2. MATERIALS AND METHODS

2.1. Sample collection

Tuna samples were obtained from various sources, including a series of samplings in Toyama, Sakaiminato, Nachi-Katsuura, Yaizu, Makurazaki, and Yamakawa fishing ports in Japan, and Venice in Los Angeles, USA, and from scientific research cruises using research vessels and chartered commercial long-line vessels. Specimens were also obtained via the scientific observer program onboard Japanese commercial long-line vessels. The samples were collected from 2010 to 2019. The sample details are shown in Table S1 in the Supplement at www.int res.com/articles/suppl/m659p175_supp.xlsx. Skipjack tuna *Katsuwonus pelamis* (SKJ) was collected as an outgroup in the ancestral character-state reconstruction analysis.

2.2. Phenotypic sex identification by visual inspection of the gonads

The sex of individuals with developed testes or ovaries was easily identified by macroscopic inspection. The small, circular sperm duct or large, saccular ovarian cavity was clearly visible in a cross section of the gonad (Fig. 1A). Histological observation of undeveloped gonads was performed using an optical microscope, because macroscopic sex identification was difficult (Fig. 1B). First, we identified either a small sperm duct and seminal lobules in the testis or a large ovarian cavity and cystic ovarian lamellae in the ovary. Testes and ovaries were then cut into small pieces and fixed in 10% neutral buffered formalin for

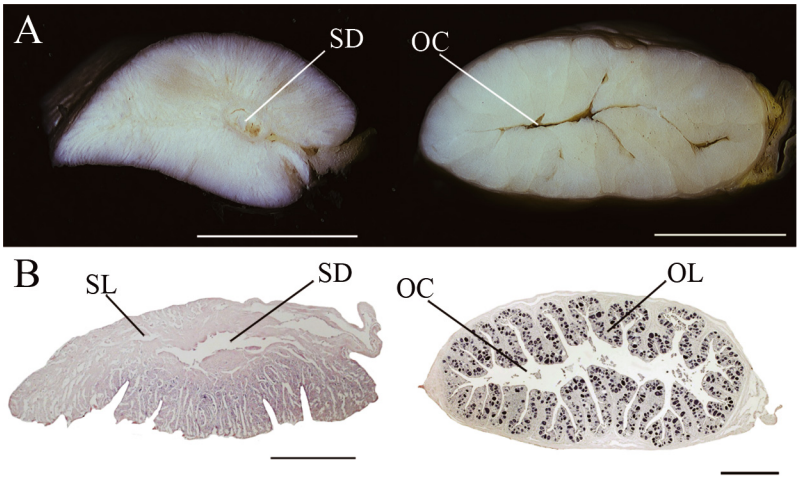

Fig. 1. Cross-sections of the testis (left) and ovary (right) of albacore. (A) Gonads extracted from a fish with a fork length (FL) of 83 cm; scale bars = 5 mm. (B) Optical micrographs of the gonads extracted from a fish with an FL of 60 cm; scale bars = 0.5 mm. SD: sperm duct; SL: seminal lobule; OC: ovarian cavity; OL: ovarian lamella

several days. The fixed gonads were gradually dehydrated in a series of ethanol solutions (60, 70, 80, 90, and 100%), and then embedded in paraffin wax and sectioned to approximately 7 µm thickness. The sectioned samples were stained with Mayer's hematoxylin and 1% eosin. The phenotypic sex identification of gonads of ALB, BET, and YFT was conducted twice by 2 researchers independently, except for the specimens mentioned below. The sex of samples obtained from scientific observers was identified by macroscopic inspection of the gonads onboard commercial vessels. If an observer could not identify the sex of a specimen, it was later identified by a researcher from images of the gonad cross section taken by the observer. The sex of PBF specimens was identified by macroscopic inspection of the gonads by local experts at Sakaiminato. For LOT, 28 sex-unidentified specimens were used to supplement the low number of sex-identified specimens of this species.

2.3. Genotypic sex identification using the male-specific DNA marker assay

A total of 490 phenotypically sex-identified tunas and the 28 sex-unidentified LOT specimens were used. Approximately 100 mg of the muscle, whole blood, or gonad tissue was placed in 1 ml of TNES-6M urea buffer (Asahida et al. 1996), treated with 20 µl of 20 mg ml^{-1} Proteinase K to release nucleic acids, and incubated for several days at room temperature. A sub-sample of the preserved lysate was purified using the DNeasy Blood & Tissue kit (Qiagen) or the MagMax Core kit (Thermo Fisher Scientific) with RNase A treatment. The purified DNA was stored at −20°C before polymerase chain reaction (PCR). The PCR assays were performed using 3 primer pairs (I–III; Suda et al. 2019) that were designed with 3′ terminal matches to PBF male-specific nucleotide sequences. Owing to a large difference in the amplification efficiency depending on whether the 3′ terminal primer sequence matches or mismatches the template DNA, the target amplicon can be detected in male samples, but not in females. An internal positive control (IPC) was amplified in both sexes to eliminate the possibility of false negatives due to PCR failure. PCR amplification was conducted using the TaKaRa Taq HS Perfect Mix with the following 2-step PCR protocol: 35 cycles for 5 s at 94°C and 20 s at 65°C. This thermal cycling profile was common to the 3 primer pairs. The PCR mixture of 10 or 20 µl contained approximately 0.5–10 ng $µl^{-1}$ purified DNA, 1× TaKaRa *Taq* HS Perfect Mix, 0.25 µM of a male-specific primer pair, and 0.025 µM of a modified MiFish-tuna primer pair (forward: 5′-TGT CCT TCC TCC TTA TCG GCT G-3′, reverse: 5′-TTG CCA GTG GCA GCT ACG ATC-3′; originally designed by Miya et al. 2015) as an IPC (224 bp). For SKJ, a modified MiFish-u primer pair (forward: 5′-GTC GGT AAA ACT CGT GCC AGC-3′, reverse: 5′-CAT AGT GGG GTA TCT AAT CCC AGT TTG-3′; originally designed by Miya et al. 2015) was used as an IPC (206 bp). All primer sequences are shown in Table A1 in the Appendix. Some primary analyses conducted together with a re-analysis (see Section 4) were performed with reaction mixtures (final volume: 20 µl) to minimize pipetting error and for convenience. Fragment analysis was performed on a Fragment Analyzer (Agilent) according to the manufacturer's instructions using the dsDNA 905 Reagent Kit. To evaluate the effectiveness of the primers for sex identification, the following indexes were calculated: sensitivity (number of true positives/number of true positives and false negatives), specificity (number of true negatives/number of true negatives and false positives), positive predictive value (number of true positives/number of positive calls), negative predictive value (number of true negatives/

number of negative calls), effectiveness (number of true positives and true negatives/number of positive and negative calls).

2.4. Examination of the sex ratio and sexual size dimorphism in ALB

A total of 209 ALB individuals were used for a practical test of the sex-identification marker. These specimens were collected from the Atlantic, Indian, and Pacific Oceans via the scientific observer program onboard Japanese commercial long-line vessels during the 2018 fiscal year. Body size (fork length, FL) was measured by the observers onboard. PCR amplification with primer pair II and the fragment analysis were conducted as described above. The body size differences between the sexes were tested using the exact Wilcoxon–Mann–Whitney (WMW) test in the R package 'coin' (Hothorn et al. 2006). The overall sex ratio of the samples was calculated, and a chi-squared test was used to examine the differences from the expected 1:1 ratio. Statistical tests were 2-tailed and the significance level was set at $\alpha = 0.05$. Statistical analyses were performed in R version 3.6.2 (R Core Team 2019).

2.5. ASR

The phylogenetic pattern of male-heterozygous polymorphisms examined using primer pair II was investigated by ancestral character-state reconstruction using the maximum likelihood method in Mesquite 3.6 (www.mesquiteproject.org, accessed 15 July 2019). Maximum likelihood reconstruction was performed using a single-rate Mk likelihood model. The character state was traced onto the phylogenetic tree topology estimated from genome-wide nuclear markers from previous studies (Díaz-Arce et al. 2016, Ciezarek et al. 2019). Although a single topology was generated in these previous genome-wide studies, the branch lengths differed depending on the dataset; therefore, only the topology was used for the present ASR.

3. RESULTS

3.1. Phenotypic sex identification

Phenotypic sex identification was successful for 490 individuals (Table 1; Table S1), but it was inconclusive for 59 individuals of ALB, BET, and YFT, because the sex identified by the 2 researchers was different. The body size of the samples that could not be sexed ranged from 56 to 73 cm FL in ALB, 40 to 42 cm standard length (SL) in BET, and 31 to 45 cm SL in YFT.

3.2. Cross-species amplification

The assays using primer pairs I and II produced the expected fragment patterns (Fig. 2A,B). The expected 149 bp male/female consensus fragments with primer pair III were not amplified in both sexes

Table 1. Amplification results of male-specific PCR using the 3 primer pairs. The size and pattern (male-specific or consensus) of amplicons are those expected in Pacific bluefin tuna *Thunnus orientalis* (PBF), for which the primer pairs were originally developed. The numbers on the left and right of the slash represent amplification success and phenotypically sexed sample, respectively. BFT: Atlantic bluefin *T. thynnus*; SBF: southern bluefin *T. maccoyii*; ALB: albacore *T. alalunga*; BET: bigeye *T. obesus*; YFT: yellowfin *T. albacares*; LOT: longtail *T. tonggol*; BLF: blackfin *T. atlanticus*; SKJ: skipjack tuna *Katsuwonus pelamis*. Numbers in parentheses were estimated from sex-unidentified samples (see Table S1 in the Supplement at www.int-res.com/articles/suppl/m659p175_supp.xlsx)

Species	Sex	Primer pair			
		I (113 bp)	II (143 bp) (Male-specific)	III (142 bp)	III (149 bp) (Consensus)
PBF	Male	12/12	12/12	12/12	12/12
	Female	0/12	0/12	0/12	12/12
BFT	Male	21/24	21/24	21/24	24/24
	Female	1/24	1/24	1/24	24/24
SBF	Male	11/45	11/45	11/45	45/45
	Female	3/48	3/48	3/48	48/48
ALB	Male	46/46	46/46	46/46	0/46
	Female	6/59	0/59	3/59	0/59
BET	Male	20/48	46/48	18/48	45/48
	Female	2/53	6/53	2/53	52/53
YFT	Male	29/32	29/32	29/32	0/32
	Female	0/32	0/32	0/32	0/32
LOT	Male	2/2 (15/15)	2/2(15/15)	2/2 (15/15)	0/2 (1/15)
	Female	0/4 (0/13)	0/4 (0/13)	0/4 (0/13)	0/4 (0/13)
BLF	Male	14/14	14/14	14/14	0/14
	Female	0/6	0/6	0/6	0/6
SKJ	Male	0/12	0/12	0/12	12/12
	Female	0/12	0/12	0/12	12/12

of ALB, YFT, LOT, and BLF (Fig. 2C). The observed amplicon sizes were slightly larger than the expected sizes. An approximately 180 bp non-target fragment was also observed (Fig. 2C). The causes of the size difference and non-target fragment are unknown, but they were also observed by Suda et al. (2019), who developed the primer pair. Therefore, we do not consider these as issues because they did not have a significant effect on sex-identification using the primer pair.

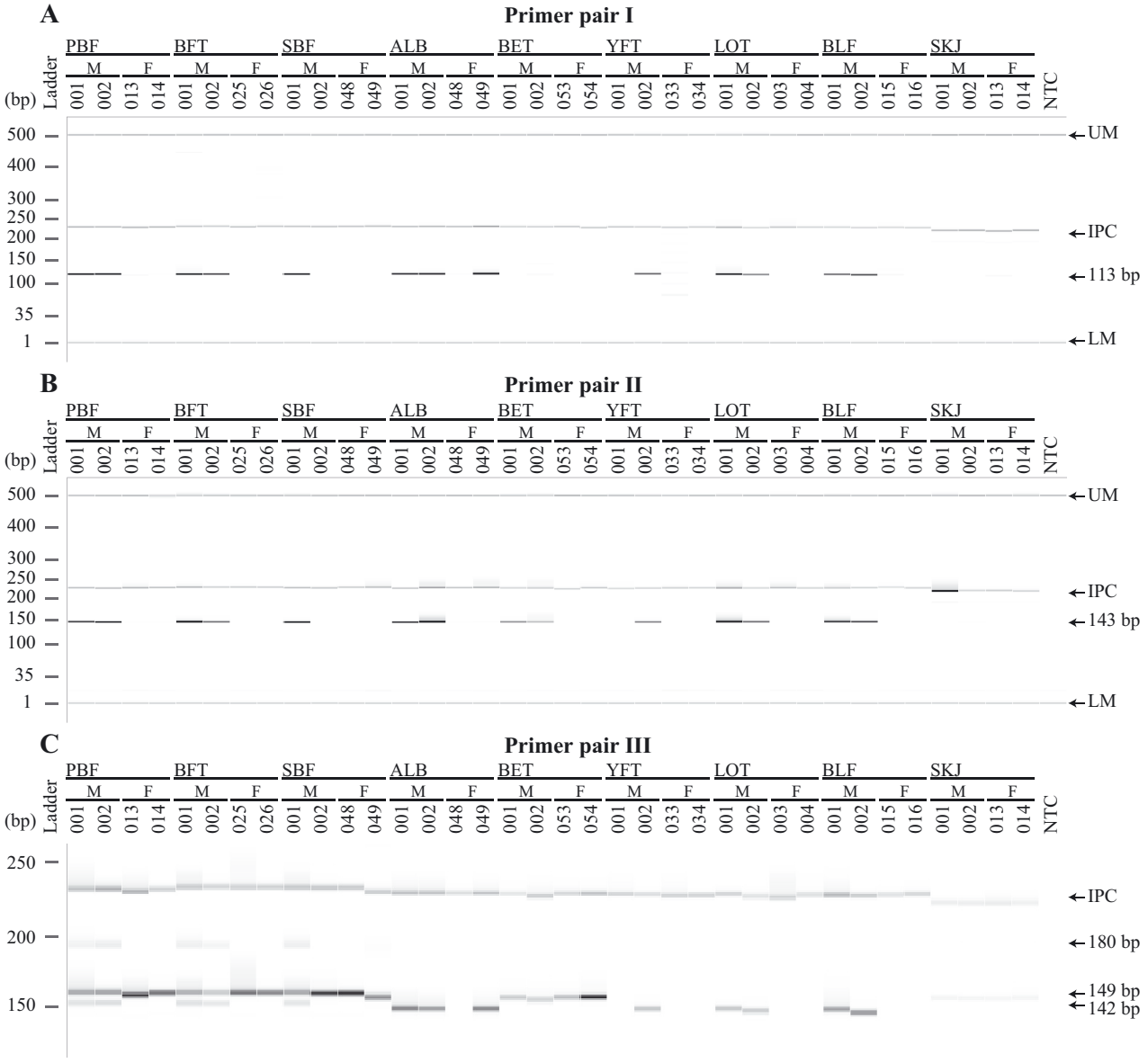

Fig. 2. Representative cross-species amplification of 3 candidate male-specific primer pairs in tunas. (A) A 113 bp male-specific amplicon was expected with primer pair I. (B) a 143 bp male-specific amplicon was expected with primer pair II. (C) 142 bp male-specific and 149 bp common amplicons were expected with primer pair III. Internal positive control (IPC) amplicons (224 or 206 bp) appeared in all lanes with the same PCR conditions. A ~180 bp fragment in (C) is a non-target amplicon that is already known to appear in male Pacific bluefin tuna. These fragment analyses were integrated across multiple runs using 12 parallel capillaries in the Fragment Analyzer. There were slight variations in the degree of migration owing to the differences in the capillary conditions and PCR amplification efficiency. The gel images in (C) were cropped and magnified for visualization. The upper marker (UM: 500 bp), amplicon sizes expected in Pacific bluefin tuna, and lower marker (LM: 1 bp) are indicated with arrows on the right. The virtual ladder marker that integrated across multiple runs is shown on the left. The no-template controls (NTCs) are shown on the right. Tuna abbreviations as in Table 1; M: male; F: female. The numbers in each lane indicate the sample ID of each species (details shown in Table S1)

3.3. Genotypic sex identification and effectiveness of the assays

The IPC fragment was successfully amplified in 513 individuals, but it was not detected in 5 individuals. The samples amplified without the IPC were excluded from the calculations of marker effectiveness. The results of amplification of the 3 candidate markers in all species and their sex-identification effectiveness are shown in Tables 1 & 2 and Table S1. For some individuals (4 BFT, 4 BET, 3 YFT, and 35 SBF), the genotypic sex was inconsistent with their phenotypic sex with all 3 candidate markers. For SBF, the genotypic sex identified with all 3 markers differed from the phenotypic sex for 34 of the 45 phenotypic males and 1 of the 48 phenotypic females.

3.4. Practical test

In all 209 ALB individuals tested, the IPC fragment amplification and genotypic sex identification were successful. The body size (FL) ranged between 80 and 113 cm in males and between 79 and 108 cm in females. Fig. 3 plots body sizes for each sex and fishing area. In all comparisons, the mean body size of males was higher than that of females. The WMW test results presented low p-values, although the values of Indian ($p = 0.05$) and Pacific Ocean samples ($p = 0.09$) were not significant (Fig. 3). The overall sex ratio of the ALB samples caught by Japanese commercial long-line vessels significantly deviated from the expected 1:1, with a dominance of males (1:1.4; chi-squared test, $p = 0.02$).

Table 2. Sensitivity (number of true positives / number of true positives and false negatives), specificity (number of true negatives / number of true negatives and false positives), positive predictive value (PPV; number of true positives / number of positive calls), negative predictive value (NPV; number of true negatives / number of negative calls), and effectiveness (number of true positives and true negatives / number of positive and negative calls) of sex identification using male-specific markers. Tuna abbreviations as in Table 1; na: not applicable

Species	Primer pair	Sensitivity	Specificity	PPV	NPV	Effectiveness
PBF	I	100	100	100	100	100
	II	100	100	100	100	100
	III	100	100	100	100	100
BFT	I	87.5	95.8	95.5	88.5	91.7
	II	87.5	95.8	95.5	88.5	91.7
	III	87.5	95.8	95.5	88.5	91.7
SBF	I	24.4	93.8	78.6	57.0	60.2
	II	24.4	93.8	78.6	57.0	60.2
	III	24.4	93.8	78.6	57.0	60.2
ALB	I	100	89.8	88.5	100	94.3
	II	100	100	100	100	100
	III	100	94.9	93.9	100	97.1
BET	I	41.7	96.2	90.9	64.6	70.3
	II	95.8	88.7	88.5	95.9	92.1
	III	37.5	96.2	90.0	63.0	68.3
YFT	I	90.6	100	100	91.4	95.3
	II	90.6	100	100	91.4	95.3
	III	90.6	100	100	91.4	95.3
LOT	I	100	100	100	100	100
	II	100	100	100	100	100
	III	100	100	100	100	100
BLF	I	100	100	100	100	100
	II	100	100	100	100	100
	III	100	100	100	100	100
SKJ	I	0	100	na	50	50
	II	0	100	na	50	50
	III	0	100	na	50	50

3.5. Ancestral character-state reconstruction

The male-heterozygous state was assigned as either present or absent for all 260 male tunas. The ASR of the male-heterozygous state revealed a relatively simple evolutionary scenario in tunas (Fig. 4), with a high probability that the male-heterozygous state was present in the common ancestor of *Thunnus*, but absent in the ancestor of SBF.

4. DISCUSSION

4.1. Difficulty in sex identification by visual gonad inspection

The phenotypic sex of 59 individuals could not be identified by visual gonad inspection. These individuals were obviously smaller than the minimum size at sexual maturity, i.e. 78 cm FL for ALB in the western North Pacific (Chen et al. 2010), 102 cm FL for BET in the eastern and Central Pacific (Schaefer et al. 2005), and 73 cm FL for YFT in western Central Pacific (Itano 2000); therefore, these individuals were assumed to be sexually immature. This confirmed that visual inspection of the gonads is not an effec-

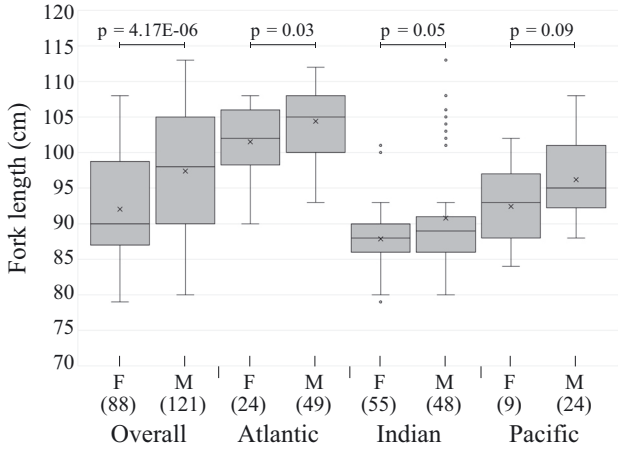

Fig. 3. Comparison of fork length between the sexes in albacore *Thunnus alalunga*. M: male; F: female. Each box plot indicates the median (center line), mean (×), interquartile range from 25 to 75% of the corresponding data (box), minimum and maximum (whiskers) and outliers (dots). Statistical significance is indicated with the p-value (exact Wilcoxon–Mann–Whitney test). Sample size is shown in parentheses

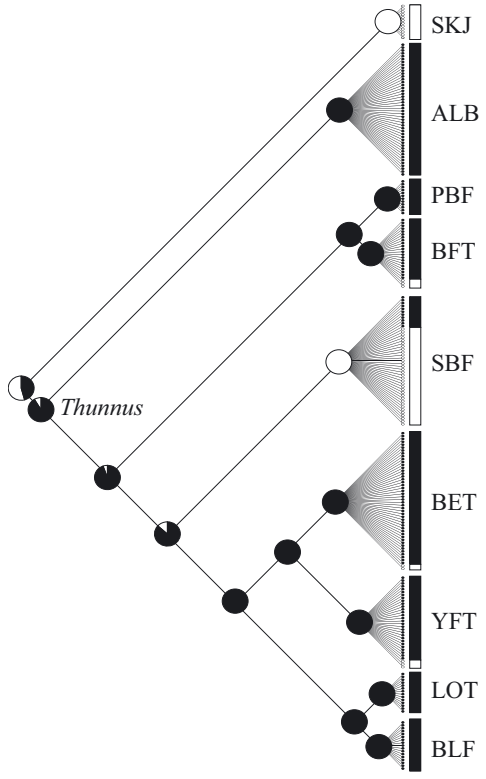

Fig. 4. Reconstructed ancestral state of male-heterozygous polymorphism. The pie charts at each node indicate the relative likelihood of presence (black portion) and absence (white portion) of the male-heterozygous state. The very small circles at each terminal node indicate the presence (black) or absence (white) of the male-heterozygous state in individual fish. Each bar plot indicates the proportion of species with the male-heterozygous state. Tuna abbreviations as in Table 1

tive sex-identification method for immature individuals. Alternative methods that are not reliant on histological observation would be advantageous, such as the vitellogenin level (e.g. Susca et al. 2001, Micera et al. 2010), but this would only be possible for a limited season.

4.2. Effectiveness of male-specific DNA markers

We applied the sex-identification markers developed for PBF (Suda et al. 2019) to other tuna species. The genotypic sex coincided with the phenotypic sex for almost all specimens, except SBF and SKJ. Primer pair II showed the highest effectiveness (91.75–100.00%) in the sex identification of tunas, except SBF and SKJ, compared with primer pair I (70.30–100.00%) and primer pair III (68.30–100.00%). Rapid, accurate, practical sex-identification methods can be applied at all life stages, including from the fertilized egg stage to the adult stage, processed products, and throughout the years regardless of the gonad development stage. Compared with visual inspection of the gonads, this method of employing DNA markers provides more effective biological information for tuna stock assessments, including sex ratio, sexual dimorphisms in growth rate, mortality, body size, and spatiotemporal distribution of wild tuna populations.

Using the DNA markers, sex identification was not successful in SBF and SKJ owing to low amplification of the male-specific markers (24.4% in SBF, 0.0% in SKJ). For the other 396 tuna specimens, excluding SBF and SKJ, the male-specific amplicons of primer pairs I, II, and III comprised 12, 10, and 9 false positives and 80, 54, and 82 false negatives, respectively. Plausible reasons for these discordances are misidentification of phenotypic sex, cross contamination, meiotic recombination, allele dropout/dropin, and sex reversal. The misidentification of phenotypic sex is unlikely to occur frequently, because 2 independent observations were conducted with immature samples, and the samples with conflicting identification results were excluded from further analyses. Furthermore, the likelihood of misidentification of phenotypic sex of samples from scientific observers onboard commercial vessels was very low because almost all fish were larger than the minimum size at sexual maturity (minimum 102 cm FL for all species). If female DNA samples were contaminated with male DNA samples, then male-specific amplifications would be observed in females as a false positive, but not vice versa. The discordances observed in

our study were more frequently false negatives than false positives; hence, there was no evidence of cross contamination. Moreover, no-template controls were not amplified (data not shown).

A change in haplotype structure among the 3 male-specific markers is possible. In PBF, the 3 markers occur in 1 linkage disequilibrium (LD) block, where recombination is extensively suppressed in a scaffold (Suda et al. 2019); however, it is not known whether the markers are in a single LD block in other tunas. These markers are expected to be present in 1 LD block because the amplification results were consistent among markers except in ALB and BET. In contrast, the different markers presented conflicting amplification results in ALB and BET, and this suggested that recombination among the markers could occur in these species. Sequence variations such as mutation, insertion, and deletion in the primer-binding sites within a species might also provide conflicting amplification results. Allele (or locus) dropout/dropin occurs when 1 or both allele(s) of a heterozygote fail(s) to PCR amplify or is/are a false positive, which may be due to either sequence-independent factors or allele-specific sequence variation. Most allele dropouts/dropins are due to sequence-independent factors, and almost all sequence-independent allele dropouts/dropins can be resolved by a re-analysis of the sample with the same assay under the same conditions (Blais et al. 2015). Clinical genotyping assays of 123 076 independent PCRs in a human disease study showed that a re-analysis with the same assay under the same conditions resolved 94% of allele dropout or dropin cases (Blais et al. 2015). In this study, a re-analysis of the majority of the discordant samples only partially resolved some discordances. Sequence independent allele dropout/dropin could reasonably explain some of the rare false negatives and false positives in this study (e.g. in ALB), but this is not a likely explanation for the frequent false negatives, for example, in SBT. These frequent false negatives might be caused by mutations in the primer-binding sites. The lack of male-specific amplification in the SKJ samples suggests that the primer-binding sites might not be present in this species.

Sex reversal is another plausible explanation, which should not be ignored, particularly for individuals of BFT, BET, and YFT that showed complete discordance between phenotypic and genotypic sex identification with all 3 markers. Sex reversal in wild fish populations is not infrequent (reviewed by Baroiller & D'Cotta 2016). For example, an extensive survey of 3004 wild-caught and wild-stock medaka *Oryzias latipes* in Japan, Korea, China, and Taiwan showed that 1.7% of phenotypic females were identified as genotypic males (XY females) and 1% of phenotypic males were identified as genotypic females (XX males) (Shinomiya et al. 2004). Similar phenomena have been reported in the chinook salmon *Oncorhynchus tshawytscha* (Cavileer et al. 2015) and pejerrey *Odontesthes hatcheri* (Yamamoto et al. 2014). To our knowledge, this study might be the first to report sex reversal in wild BFT, BET, and YFT. However, further research, including analysis of sex-determination genes or regions, is necessary to clarify whether sex reversal occurs in wild tunas.

4.3. Practical test of sex ratio and sexual size dimorphism in ALB

Japanese commercial long-line vessels fish for ALB in 3 oceans. The science observer program of Japan collects some biological data of fish caught by the Japanese commercial long-line vessels. However, sexual data could not be collected for ALB, in contrast to those for other tuna species, because ALB individuals were not gutted onboard. The sex-specific variation in the growth of ALB is an essential biological parameter for modeling stock population dynamics. Sexual size dimorphism, where adult males attain a larger size than females, was reported in ALB 6 decades ago (e.g. Otsu & Uchida 1959, Chen et al. 2012), and a male-biased sex ratio in large ALB was reported in the Mediterranean (>75 cm FL; Saber et al. 2015) and North Pacific (>100 cm FL; Chen et al. 2010). Although sex-specific growth curves based on the sex-specific size variation (ISC 2017) have been adopted in the stock assessment of North Pacific ALB, details of sexual differences in growth remain unknown owing to uncertainties in the sex identification of juveniles and/or products of ALB. The results of our practical test of genotypic sex identification in samples of ALB larger than 79 cm FL caught by Japanese long-line vessels were consistent with the sexual size dimorphism and male-biased sex ratio reported previously (Chen et al. 2010, 2012). Owing to the small number of samples and the possibility of sampling bias, our data cannot reliably represent phenomena such as the sex ratio and sexual size dimorphism of wild or fished populations. However, the assays can reveal these phenomena if sufficient numbers of adult and juvenile specimens are available. This result demonstrates that the genotypic sex-identification methods are applicable for biological studies, such as identifying the sex of juveniles, and for stock studies.

4.4. Evolution of sex-associated genomic regions in tunas

As all cross-species amplifications (excluding SBF) of the male-specific markers developed for PBF clearly indicated male-heterozygous polymorphisms, it is likely that a male-heterozygous sex-associated region exists in the *Thunnus* genome. The ASR showed that the male-heterozygous state was present in the genome of a common ancestor before the evolution of the genus. This state has been well maintained among ancestral lineages such as ALB, PBF, and BFT (Fig. 4). In these species, the high effectiveness of the sex-identification markers was well supported. In contrast, SBF was thought to have lost the state during evolution. However, because the state was present in one-quarter of SBF males, there is a possibility that mutations in the primer-binding sites may have prevented the detection of the state. Although loss of the state was observed at a low frequency in the derived lineages such as BET and YFT (Fig. 4), it was not phylogenetically lost, and we assume that the state was not detected owing to individual variations in the primer-binding sites. Our study indicates that other tuna species may have male-heterogametic SD systems similar to that in PBF. Heteromorphic sex chromosomes and SD genes or regions have not hitherto been found in tunas (Ratty et al. 1986, Ida et al. 1993, Agawa et al. 2015, Lee et al. 2018, Suda et al. 2019). Although 250 sex-associated SNPs have been identified from 15 scaffolds in the PBF genome (Suda et al. 2019), these scaffolds could be combined into a single chromosome with future high-quality genome assembly. Therefore, in the future, studies such as chromosome level assembly, transcriptome, and expression analyses, in addition to comparative genomics among tuna species, are required to clarify the SD systems and genes or regions of tunas.

Acknowledgements. We thank K. Kobayashi (Wakayama Prefectural Fisheries Station), H. Ichihara (Miyazaki Prefecture Fisheries Research Institute), and A. Kimoto (The International Commission for the Conservation of Atlantic Tunas) for helping with the collection of fishes; Y. Ozaki and M. Murakami (National Research Institute of Far Sea Fisheries, Japan: NRIFSF) for helping in dissection of fishes; H. Ashida (NRIFSF) for technical advice in histological observation; M. Michibayashi, H. Hasegawa, and H. Kawamura-Ichikawa (NRIFSF) for helping with the molecular experiment; Y. Hayashi (NRIFSF) for curation of specimen data; and T. Ito and Y. Tsuda (NRIFSF) for their constructive comments on an earlier draft. This work was financially supported by the Japan Fisheries Agency.

LITERATURE CITED

Agawa Y, Iwaki M, Komiya T, Honryo T and others (2015) Identification of male sex-linked DNA sequence of the cultured Pacific bluefin tuna *Thunnus orientalis*. Fish Sci 81:113–121

Asahida T, Kobayashi T, Saitoh K, Nakayama I (1996) Tissue preservation and total DNA extraction from fish stored at ambient temperature using buffers containing high concentration of urea. Fish Sci 62:727–730

Ashida H, Suzuki N, Tanabe T, Suzuki N, Aonuma Y (2015) Reproductive condition, batch fecundity, and spawning fraction of large Pacific bluefin tuna *Thunnus orientalis* landed at Ishigaki Island, Okinawa, Japan. Environ Biol Fishes 98:1173–1183

Baroiller JF, D'Cotta H (2016) The reversible sex of gonochoristic fish: insights and consequences. Sex Dev 10: 242–266

Blais J, Lavoie SB, Giroux S, Bussières J and others (2015) Risk of misdiagnosis due to allele dropout and false-positive PCR artifacts in molecular diagnostics analysis of 30,769 genotypes. J Mol Diagn 17:505–514

Cavileer TD, Hunter SS, Olsen J, Wenburg J, Nagler JJ (2015) A sex-determining gene (*sdY*) assay shows discordance between phenotypic and genotypic sex in wild populations of chinook salmon. Trans Am Fish Soc 144: 423–430

Chen KS, Crone PR, Hsu CC (2010) Reproductive biology of albacore *Thunnus alalunga*. J Fish Biol 77:119–136

Chen KS, Shimose T, Tanabe T, Chen CY, Hsu CC (2012) Age and growth of albacore *Thunnus alalunga* in the north Pacific Ocean. J Fish Biol 80:2328–2344

Ciezarek AG, Osborne OG, Shipley ON, Brooks EJ and others (2019) Phylotranscriptomic insights into the diversification of endothermic *Thunnus* tunas. Mol Biol Evol 36: 84–96

Díaz-Arce N, Arrizabalaga H, Murua H, Irigoien X, Rodríguez-Ezpeleta N (2016) RAD-seq derived genome-wide nuclear markers resolve the phylogeny of tunas. Mol Phylogenet Evol 102:202–207

Farley JH, Clear NP, Leroy B, Davis TLO, McPherson G (2006) Age, growth and preliminary estimates of maturity of bigeye tuna, *Thunnus obesus*, in the Australian region. Mar Freshw Res 57:713–724

Hothorn T, Hornik K, van de Wiel MA, Zeileis A (2006) A Lego system for conditional inference. Am Stat 60: 257–263

Ida H, Oka N, Terashima H, Hayashizaki K (1993) Karyotypes and cellular DNA contents of three species of family Scombridae from Japan. Bull Jpn Soc Sci Fish 59: 1319–1323

ISC (International Scientific Committee for Tuna and Tuna-like Species in the North Pacific Ocean) (2017) Report of the albacore working group. Stock assessment of albacore tuna in the north Pacific Ocean 2017. ISC, Vancouver

Itano DG (2000) The reproductive biology of yellowfin tuna (*Thunnus albacares*) in Hawaiian waters and the western tropical Pacific Ocean: project summary. SOEST 00-01 JIMAR Contribution 00-328. Pelagic Fisheries Research Program, JIMAR, University of Hawaii, Manoa, HI

Lee YH, Yen TB, Chen CF, Tseng MC (2018) Variation in the karyotype, cytochrome *b* gene, and 5s rDNA of four *Thunnus* (Perciformes, Scombridae) tunas. Zool Stud 57: e34

Lin Y, Tzeng W (2010) Sexual dimorphism in the growth rate of southern bluefin tuna *Thunnus maccoyii* in the Indian Ocean specimen collection. Taiwan Shuichanxue Hui Kan 37:135–151

Micera E, Zupa R, Zarrilli A, Camarda A and others (2010) A rapid latex agglutination test for gender identification in the Atlantic bluefin tuna, *Thunnus thynnus* (Linnaeus). Aquac Res 41:1396–1401

Miya M, Sato Y, Fukunaga T, Sado T and others (2015) MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. R Soc Open Sci 2:150088

Okochi Y, Abe O, Tanaka S, Ishihara Y, Shimizu A (2016) Reproductive biology of female Pacific bluefin tuna, *Thunnus orientalis*, in the Sea of Japan. Fish Res 174: 30–39

Otsu T, Uchida RN (1959) Study of age determination by hard parts of albacore from central north Pacific and Hawaiian waters (Fish Bull 150). Fish Bull 59:353–363

Penman DJ, Piferrer F (2008) Fish gonadogenesis. I. Genetic and environmental mechanisms of sex determination. Rev Fish Sci 16:16–34

R Core Team (2019) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna

Ratty FJ, Song YC, Laurs RM (1986) Chromosomal analysis of albacore, *Thunnus alalunga*, and yellowfin, *Thunnus albacares*, and skipjack, *Katsuwonus pelamis*, tuna. Fish Bull 84:469–476

Saber S, Ortiz De Urbina J, Gómez-Vives MJ, Macías D (2015) Some aspects of the reproductive biology of albacore *Thunnus alalunga* from the western Mediterranean Sea. J Mar Biol Assoc UK 95:1705–1715

Santamaria N, Bello G, Corriero A, Deflorio M, Vassallo-Agius R, Bök T, De Metrio G (2009) Age and growth of Atlantic bluefin tuna, *Thunnus thynnus* (Osteichthyes: Thunnidae), in the Mediterranean Sea. J Appl Ichthyol 25:38–45

Schaefer KM, Fuller DW, Miyabe N (2005) Reproductive biology of bigeye tuna (*Thunnus obesus*) in the eastern and central Pacific Ocean. Inter-Am Trop Tuna Comm Bull 23:3–31

Schartl M (2004) Sex chromosome evolution in non-mammalian vertebrates. Curr Opin Genet Dev 14:634–641

Shih CL, Hsu CC, Chen CY (2014) First attempt to age yellowfin tuna, *Thunnus albacares*, in the Indian Ocean, based on sectioned otoliths. Fish Res 149:19–23

Shimose T, Aonuma Y, Suzuki N, Tanabe T (2016) Sexual differences in the occurrence of Pacific bluefin tuna *Thunnus orientalis* in the spawning ground, Yaeyama Islands. Environ Biol Fishes 99:351–360

Shinomiya A, Otake H, Togashi K, Hamaguchi S, Sakaizumi M (2004) Field survey of sex-reversals in the medaka, *Oryzias latipes*: genotypic sexing of wild populations. Zool Sci 21:613–619

Suda A, Nishiki I, Iwasaki Y, Matsuura A, Akita T, Suzuki N, Fujiwara A (2019) Improvement of the Pacific bluefin tuna (*Thunnus orientalis*) reference genome and development of male-specific DNA markers. Sci Rep 9:14450

Susca V, Corriero A, Bridges CR, De Metrio G (2001) Study of the sexual maturity of female bluefin tuna: purification and partial characterization of vitellogenin and its use in an enzyme-linked immunosorbent assay. J Fish Biol 58: 815–831

Takehana Y, Naruse K, Hamaguchi S, Sakaizumi M (2007) Evolution of ZZ/ZW and XX/XY sex-determination systems in the closely related medaka species, *Oryzias hubbsi* and *O. dancena.* Chromosoma 116:463–470

Williams AJ, Farley JH, Hoyle SD, Davies CR, Nicol SJ (2012) Spatial and sex-specific variation in growth of albacore tuna (*Thunnus alalunga*) across the South Pacific Ocean. PLOS ONE 7:e39318

Yamamoto Y, Zhang Y, Sarida M, Hattori RS, Strüssmann CA (2014) Coexistence of genotypic and temperature-dependent sex determination in pejerrey *Odontesthes bonariensis.* PLOS ONE 9:e102574

Appendix.

Table A1. PCR primers for sex-identification and an internal positive control, the NADH dehydrogenase subunit 5 gene. SKJ: skipjack tuna

Primer pair	Name	Sequence (5′ to 3′)	Size (bp)	Original reference
Pair I	sca64_3724604_F sca64_3724604_R	TGCACCTGTAACTCACTAACCG CCTTTTCCTGGCCTCTTTACAT	113	Suda et al. (2019)
Pair II	sca64_3726411_F sca64_3726411_R	GCAGACAAAAAGCCATTCG CTGATGWCCTCTGTAACACAATCAT[a]	143	Suda et al. (2019)
Pair III	sca64_3724591_F sca64_3724591_R	CAGAAATCACCAGTGCACC GGATATTATTAGGAAACCTTTTCCTG	142 and 149	Suda et al. (2019)
Modified MiFish-tuna Internal positive control (IPC)	modified-MiFish-tuna-ND5-F modified-MiFish-tuna-ND5-Rs	ATGTCCTTCCTCCTTATCGGCTG TTGCCAGTGGCAGCTACGATC	224	Miya et al. (2015)
Modified MiFish-u Internal positive control (IPC) for SKJ	modified-MiFish-u-ND5-Fs modified-MiFish-u-ND5-Rs	GTCGGTAAAACTCGTGCCAGC CATAGTGGGGTATCTAATCCCAGTTTG	206	Miya et al. (2015)

[a]International Union of Pure and Applied Chemistry (IUPAC) nucleotide base code: W = A or T

Editorial responsibility: Philippe Borsa, Montpellier, France
Reviewed by: 3 anonymous referees

Submitted: April 3, 2020;
Accepted: November 3, 2020
Proofs received from author(s): January 19, 2021