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ORIGINAL ARTICLE **Aquaculture**

Identification of male sex-linked DNA sequence of the cultured Pacific bluefin tuna *Thunnus orientalis*

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Abstract A male-specific marker of a DNA sequence for the Pacific bluefin tuna *Thunnus orientalis*, *Male delta 6* (*Md6*), was identified by comparative study of the sequences obtained by F3-cultured male and female AFLP-selective DNA amplification products, followed by high-throughput DNA sequencing. *Md6* was characteristic in continuous 6-bp nucleotide deletions compared to redundant sequences that could be seen in both sexes. The PCR genotyping primers were developed based on the *Md6* sequence, which showed that 29 of 32 F3 males (90.6 %) and 9 of 13 (69.2 %) F2 parental males were genotyping positive, whereas in females, the genotyping test was negative in parental F2 ($n = 12$), and 1 of 32 (3.1 %) F3 female was *Md6*-positive. Parentage allocation tests suggested that *Md6*-positive F2 males were more attendant in spawning than *Md6*-negative males, suggesting that *Md6* was passed from male parents to male progeny under aquaculture conditions.

Keywords Pacific bluefin tuna · Male marker DNA sequence · Genotyping · AFLP

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Introduction

The bluefin tuna provides favorable food features for consumers, and food market fish demands have been increasing in recent years. However, their natural populations are not large enough to satisfy the need. In fact, the Atlantic bluefin tuna *Thunnus thynnus* was heavily fished in the 1990s, and despite international resource management, is now threatened with extinction [1]. Various resource conservation measures have been implemented, not only for the Atlantic bluefin tuna, but also for the Pacific bluefin tuna (PBF) *Thunnus orientalis*, such as the recent listing of the PBF by the Western and Central Pacific Fisheries Commission, in efforts to collect information on its fishing size and location in order to monitor resource conditions. In addition, the commission decided to reduce the PBF fishing level to below that from 2002 to 2004 [2]. However, effective management

of tuna natural resources is still difficult because of the lack of scientific knowledge regarding its base—for example, the genetic structure of the tuna population over the ocean, spawning ecology, and early life history.

Tuna ranching, which involves the capture of wild fish to use for seeding, has been conducted to generate a market supply, but it is completely dependent on natural resources, and may cause overfishing, as was the case with the Atlantic bluefin tuna [1]. In contrast, PBF full-life-cycle aquaculture from eggs to broodstock is not dependent on wild PBF, and is a possible strategy for conserving their natural resources [3–5]. There are still many problems remaining, however, for the establishment of full-cycle tuna aquaculture technology, among which is the prerequisite stable supply of fertilized eggs for fingerling production.

In red sea bream *Pagrus major* and striped beakfish *Oplegnathus fasciatus*, fish broodstock units are organized with a high ratio of females to males in order to produce a massive number of fertilized eggs, as female maturation often becomes a bottleneck of reproduction in cultured fishes. A female-rich broodstock is desirable in PBF aquaculture as well. The spawning season of these species under aquaculture conditions in Kushimoto, Japan, is from June through August. Most males reach sexual maturity during the season, while only 10 % of females achieve maturity [5, 6]. It has also often been observed that female PBF in our sea cage die after the spawning season. This phenomenon has also been reported in the PBF broodstock tank at the Seikai National Fisheries Research Institute in Nagasaki, Japan (personal communication), and in yellowfin tuna (YFT, *Thunnus albacares*) at the Inter-American Tropical Tuna Commission (IATTC) Achotines Laboratory in the Republic of Panama (personal communication). Therefore, it is desirable to establish a female-enriched PBF broodstock [6]. It is difficult to create a PBF broodstock with a high ratio of females to males because there are no visible differences in their external morphology or in chromosomal features between the sexes in this fish [7]. The sexing of mature female PBFs is practicable by immunological detection of vitellogenin, which is an egg yolk precursor protein expressed in the females [8]. It is also possible to analyze the expression of the vitellogenin gene in the liver and ovaries [9], but the manipulation for a histological examination of internal organs in these sexing sacrifices the treated fish, who die or are greatly damaged. Even if sexing is possible, the grouping of mature tuna is very difficult because of their large body, and this results in a heavy loss of fish. Therefore, new techniques are needed for tuna sexing, and genotyping by differences in DNA sequences enables the sexing of tuna at any age, especially juveniles, which are easily handled. Genotyping requires a fin tip or a drop of blood or mucus to determine the fish sex, which causes minimal damage to the tested fish.

In our previous study, we performed AFLP [10] to find PBF sex-linked DNA, and found a male characteristic fragment [11]. In this study, we report the DNA sequence of the male characteristic fragment and its heredity structure in the cultured Pacific bluefin tuna for future contribution to tuna sex distinction and broodstock management.

Materials and methods

Pacific bluefin tuna

F2 and F3 generations of the Pacific bluefin tuna were artificially hatched and then reared in the Oshima Hatchery, Fish Nursery Center, Kinki University. An ancestral wild fish was caught in 1987 on the Pacific coast of Wakayama, Japan. The PBF F1 generation was born in 1995 and 1996, and the fish cages of both lots were then brought together. The F2 generation was produced in 2002 from the F1 group [4, 12], which produced the F3 generation in 2007 [13]. The average body weight of the fish used in this study was as follows: F2 Oshima: 198.4 kg for 13 males and 151.2 kg for 12 females; F3 Oshima: 47.6 kg for 32 males and 46.2 kg for 32 females; 2005 wild: 112.9 kg for 4 males, 94.2 kg for 5 females; 2007 wild: 80.3 kg for 20 males and 78.7 kg for 20 females. All fishes were large enough for sex distinction by visual examination of the gonads.

DNA material

Whole blood of the F3 tuna was collected, and the DNA was purified based on the method described by Agawa et al. [11]. A part of a fin or the liver was collected from accidentally killed F2 fish, and the DNA was then purified using the DNeasy Blood and Tissue kit (Qiagen, Venlo, Netherlands). The DNA materials subjected to high-throughput sequencing were from one Oshima F3 male and female, and the DNA fragments were selectively amplified using AFLP, with second amplification primers of *Mse*I+CAT 5′-GAT GAGTCCTGAGTAACAT, and *Eco*RI+AGG 5′-AGACT GCGTACCAATTCAGG, which was performed according to the original protocol [10], with slight modifications in Agawa et al. [11]. The secondary selective amplification was conducted in a total volume of 360 µl in both sexes. After the reaction, the solution was ethanol-precipitated, and then reconstituted in 30 µl of TE buffer. The concentrate was mixed with 7 µl of 6× loading dye (Nippon Gene Co., Ltd., Tokyo, Japan), and then electrophoresed using a 2.3 % Agarose21 (Nippon Gene) gel at 30 V for 180 min in 1× TAE buffer. The gel was stained using 10,000-fold-diluted SYBR Green I (Takara Bio, Inc., Shiga, Japan) in 1× TAE buffer (Fig. 1), then sliced manually under an LED safe blue light (BioTools Inc., Gunma, Japan). Segments of

approximately 300–600 bp were sliced, and DNA in the gel was then purified using the QIAquick Gel Extraction Kit (Qiagen) and subjected to high-throughput sequencing.

High-throughput sequencing

An F3 male id. 3 and an F3 female id. 3 (Fig. 3) DNA were subjected to high-throughput sequencing. Briefly, AFLP products of the fishes were processed using the Rapid Library Preparation Kit (F. Hoffmann-La Roche, Basel, Switzerland) with MID (barcoded adaptors), according to the manufacturer's instructions, and resultant libraries were pyro-sequenced using GS Junior. Base-calling and sequence data filtering were performed using GS Run Processor v2.5p1 software (Roche), with default settings, and filtered sequences were subjected to the following analyses. The sequences that satisfied all of the following parameters were subjected to data acquisition: sequences contained *Mse*I+CAT or *Eco*RI+AGG primer sequence at one end, did not include uncertain Ns, and the nucleotide length was more than 380 bp. The multiple alignments of the obtained sequences were shown using Genetyx ver. 11 software (Genetyx Corporation, Tokyo, Japan).

Genotyping assay

Genotyping was performed in a reaction volume of 20 µl containing 20 ng of genomic DNA, 10 µl of 2× PCR pre-mix, Quick Taq HS DyeMix (TOYOBO Co., Ltd, Osaka, Japan), and 8.0 pmol of forward and reverse primer, with the remaining volume comprising distilled water. For the genotyping PCR of the male characteristic marker *Md6*, the PBF-Md6-F primer 5′-TTTGTTGTGTGACATGAACGAG and PBF-Md6-R primer 5′-CACACAACAGCTCCTGGACTTG were used. For the internal control PCR, primers of tuna *β-actin* F 5′-ACCCACACAGTGCCCATCTA and tuna *β-actin* R 5′-TCACGCACGATTTCCCTCT were used [14]. For *Md6* and *β-actin* PCR, the thermal conditions were programmed as follows: 2 min at 94 °C, followed by 30 cycles of 94 °C for 20 s, 60 °C for 25 s, and 72 °C for 20 s, followed by a final extension of 72 °C for 3 min. All of the thermal incubations in this study were performed in a PC818 PCR System (ASTEC Co., Ltd., Fukuoka, Japan). The oligonucleotides used in this study were purchased as Invitrogen™ (Life Technologies, Carlsbad, CA, USA).

Mitochondrial haplotype analysis

The mitochondrial haplotype was determined with partial D-loop sequencing. The PCR primers for PBF D-loop amplification were developed by Dr. E. Soeda as PBF D-loop F Soe 5′-TACCCCTAACTCCCAAAGCTAGG and PBF D-loop R Soe 5′-GCTTTCTAGGGCCCATCTTAAC. The PCR was conducted in a final volume of 20 µl containing 20 ng of genomic DNA, 10 µl of 2× PCR pre-mix, EmeraldAmp (Takara Bio, Inc.), and 8.0 pmol of forward and reverse primer, with the remaining volume comprising distilled water. The thermal conditions were programmed as follows: 2 min at 94 °C, followed by 30 cycles of 94 °C for 20 s, 60 °C for 30 s, and 72 °C for 60 s, followed by a final extension of 72 °C for 3 min. PCR products were purified using the QIAquick PCR Purification kit (Qiagen), and then subjected to BigDye labeling using the PBF D-loop F Soe primer. Labeling and sequencing were conducted by Macrogen Japan Corp. (Tokyo, Japan).

Parentage analysis

SSR loci were amplified by PCR using fluorescent *Ttho-1*, *Ttho-8*, *Ttho-21*, *Ttho-34* [15], and *CmrTa-125* primers [16], following the method reported by Nomura et al. [17]. Fragment lengths of SSR were obtained with the ABI 3730 sequencer, and then analyzed using the PARFEX for exclusion method for parentage analysis software [18]. When candidate paternal fish of the F3 male 1 were suggested as F2 male 1, 2, and 3, with the possibility of 0.2, 0.3 and 0.5 (without mismatch marker), they were scored for F2 male 1 as 0.2, 2 as 0.3 and 3 as 0.5. All F2 males were scored as noted below. The scores were then summed and shown as the total score in Tables 2 (mitochondrial haplotype a) and 3 (haplotype b).

Results

Confirmation of the male characteristic DNA fragments by common electrophoresis

In our previous screening of sex-linked DNA fragments, a male characteristic DNA fragment was observed in the AFLP second amplification product that was produced with primers for *Eco*RI+AGG and *Mse*I+CAT in the F3 males [11]. The fragment was detected in a highly sensitive DNA fragment analyzer in the previous study. In this study, a 440 bp candidate male characteristic DNA fragment, confirmed using common agarose gel electrophoresis, was observed in the F3 males but not in females (Fig. 1). The DNA fragment shown by the white dotted squares indicates the regions where the gel was subjected to DNA extraction and then high-throughput sequencing. The arrowhead indicates the male characteristic DNA fragment.

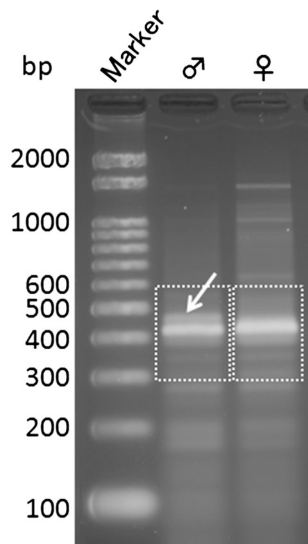

Fig. 1 Gel electrophoresis of amplified fragment length polymorphism (AFLP) secondary selective amplification products of genomic DNA for male and female Pacific Bluefin tuna. The *dotted square* indicates a region subjected to DNA extraction, followed by next-generation sequencing. The *arrowhead* indicates a band observed in male but not female AFLP products.

Fig. 2 Nucleotide sequence alignment obtained using high-throughput sequencing. Identical nucleotides are indicated by *dot*s, nucleotide deletions are indicated by *hyphen*s, and substitutions are written among the *dots* and highlighted by *black boxes*. The five most frequent sequences in both males and females are shown. A 6-bp nucleotide deletion was confirmed at position 173 in male sequence 5, and was not found in any of the female sequences (Fig. S1; Table S1). The primers used for male genotyping PCR are shown on the alignment.

Sequence analysis of the male characteristic DNA from AFLP products

A total of 35,512 independent reads were obtained from AFLP secondary selective amplification products of an F3 male (Fig. 3, male id. 3), for which perfect matched sequences were collected, and then 17,233 unique contigs were assembled. In an F3 female (Fig. 3, female id. 3), 23,559 independent reads were obtained, and 11,699 unique contigs were then assembled. The nucleotide sequence alignment of the top five most frequent sequences in both sexes were shown (Fig. 2, DDBJ Accession IDs are *AB911110* to *AB911119*), which were equal to 2,852 reads in the male and 2,835 in the female. Interestingly, male contigs in fifth most frequent sequence (*AB911114*) contained a characteristic 6-bp continuous deletion at nucleotide position 173 (Fig. 2), and we designated this sequence as *Male delta-6* (*Md6*). None of the female sequences of the 23,559 reads contained the 6-bp deletion. All of the five most frequent sequences were nearly identical, and the sequence length varied from 408 to 416 bp. One- or two-nucleotide deletions and substitutions were found throughout the sequences (Figs. 2 and S1).

To further investigate the sequences obtained (Fig. 2), a BLAST [19] search was performed. The PBF genomic sequence *BADN01109032* [20] showed the highest identity through *Md6* sequence 382 of 408 bp, with a high Expect value of 8^{e-139}. The search was also conducted for cDNA of the Atlantic bluefin tuna *Thunnus thynus* [21], and the results indicated that a probable similar sequence was *EC41822*, in which ovary transcript and nucleotide position 220–274 of *EC41822* was similar to 173–227 of *Md6*, with a low Expect value of *0.014*. In addition, the sequences had little similarity to the *Dicentrarchus labrax* chromosome sequence (*FQ310508.3*), with an Expect value of 3^{e-13}, when the BLAST search was conducted in the NCBI Somewhat Similar Sequences (blastn) program (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). The nucleotide sequences of *Md6* and redundant sequences showed no significant similarities to known coding genes, which may suggest that the sequences may not represent conserved coding genes, and rather, that the sequences had little similarity to registered microsatellites, especially in the TG repeated sequence.

The *Md6* segregation from F2 males to F3 males

Primers were designed to perform male genotyping as follows: one was designed to step over the 6-bp deletion, and another was designed to coincide with the 3′ end G/C 245 SNP of the nucleotide alignment expected to be selectable for *Md6*, as all of the sequences with the 6 bp deletion showed C245, while others showed G245 (Fig. 2; Fig S1). PCR genotyping of *Md6* was conducted in each of the 32 males and females of the F3 generation using genomic DNA as a template. In F3 fish, *Md6* genotyping showed nearly perfect concordance with the phenotypic sex (Fig. 3), suggesting that *Md6* is a part of the male sex chromosome. In addition, the genotyping results coincided with the results of the AFLP study [11]. Genotyping PCR was also conducted for the F2 broodstock fish, and showed that 9 of 13 males were *Md6*-positive, whereas all of the 12 females tested were negative (Fig. 4), indicating that *Md6* was passed from the parental male(s) to the male progenies. To confirm the genetic relationship between F2 and F3, we conducted mitochondrial haplotype analysis, which showed that three haplotypes were found in cultured F2 and five in F3 (Fig. S2, DDBJ accession IDs are *AB933578 to AB933582*), Haplotypes a and b were found in both F2 and F3, while in wild fish, 49 unique sequences were obtained from 49 wild PBF (Table 1, DDBJ Accession IDs are *AB933583 to AB933631*). These results support the idea that haplotypes a and b are familial, and therefore *Md6* was transferred from the F2 to the F3 generation.

```
>Male_Seq_1     1 AGACTGCGTACCAATTCAGGTCAACATTCAAGTGG-TAATTATGAAGTGGAAAAGCTGAAAGCTCTGATATTTACTGGAT 79
>Male_Seq_2     1 ...................................-............................................ 79
>Male_Seq_3     1 ...................................-............................................ 79
>Male_Seq_4     1 ...................................G............................................ 80
>Male_Seq_5     1 ...................................-............................................ 79
>Female_Seq_1   1 ...................................-............................................ 79
>Female_Seq_2   1 ...................................-............................................ 79
>Female_Seq_3   1 ...................................G............................................ 80
>Female_Seq_4   1 ...................................-............................................ 79
>Female_Seq_5   1 ...................................-............................................ 79

>Male_Seq_1    80 ATGCTGGATTATTGTCAATAACAGTATTTTTGAGCCTCATTCCACCAACTTTGTAATCATGCAGCCGTCTTGATTTGTTG 159
>Male_Seq_2    80 ................................................................................ 159
>Male_Seq_3    80 ...G............................................................................ 159
>Male_Seq_4    81 ...G............................................................................ 160
>Male_Seq_5    80 ................................................................................ 159
>Female_Seq_1  80 ...G............................................................................ 159
>Female_Seq_2  80 ................................................................................ 159
>Female_Seq_3  81 ...G............................................................................ 160
>Female_Seq_4  80 ................................................................................ 159
>Female_Seq_5  80 ................................................................................ 159

        5'-TTTGTTGTGTGACATGAACG          AG-3' Primer PBT-Y-F
>Male_Seq_1   160 TGTGACATGAACGCTGGCGAGTTGTAAACATGGGAATCTTTCCCGCCTCTACCATCCATCCCATATGATGTGAACACAGC 239
>Male_Seq_2   160 ................................................................................ 239
>Male_Seq_3   160 ................................................................................ 239
>Male_Seq_4   161 ................................................................................ 240
>Male_Seq_5   160 .............------............................................................. 233
>Female_Seq_1 160 ................................................................................ 239
>Female_Seq_2 160 ................................................................................ 239
>Female_Seq_3 161 ................................................................................ 240
>Female_Seq_4 160 ................................................................................ 239
>Female_Seq_5 160 ................................................................................ 239

                         3'-GTTCAGGTCCTCGACAACACAC-5' Primer PBT-Y-R
>Male_Seq_1   240 ATTAGAGATGAGAAGTCCAGGAGCTGTTGTGTGTTTCTGACATACAGGAAGTATTTAGTTTT--CTCTGGAGTGTTT-AT 316
>Male_Seq_2   240 ..............................................................-T.............T.. 318
>Male_Seq_3   240 ..............................................................TT.............-.. 318
>Male_Seq_4   241 ..............................................................--.............-.. 317
>Male_Seq_5   234 ...........C..................................................-T.............-.. 311
>Female_Seq_1 240 ..............................................................TT.............-.. 318
>Female_Seq_2 240 ..............................................................--.............-.. 316
>Female_Seq_3 241 ..............................................................--.............-.. 317
>Female_Seq_4 240 ..............................................................-T.............T.. 318
>Female_Seq_5 240 ..............................................................-T.............T.. 318

>Male_Seq_1   317 TT-AGTTCCTGTGTAGAGAATAAAGTTCATGATGATCAGTAGCTGATGTTAGTTTTCTCCATTTATTTTGTGTGTGTGCA 395
>Male_Seq_2   319 ..T............................................................................. 398
>Male_Seq_3   319 ..-............................................................................. 397
>Male_Seq_4   318 ..-............................................................................. 396
>Male_Seq_5   312 ..-............................................................................. 390
>Female_Seq_1 319 ..-............................................................................. 397
>Female_Seq_2 317 ..-............................................................................. 395
>Female_Seq_3 318 ..-............................................................................. 396
>Female_Seq_4 319 ..T............................................................................. 398
>Female_Seq_5 319 ..-............................................................................. 397

>Male_Seq_1   396 TGTTACTCAGGACTCATC 413
>Male_Seq_2   399 .................. 416
>Male_Seq_3   398 .................. 415
>Male_Seq_4   397 .................. 414
>Male_Seq_5   391 .................. 408
>Female_Seq_1 398 .................. 415
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>Female_Seq_4 399 .................. 416
>Female_Seq_5 398 .................. 415
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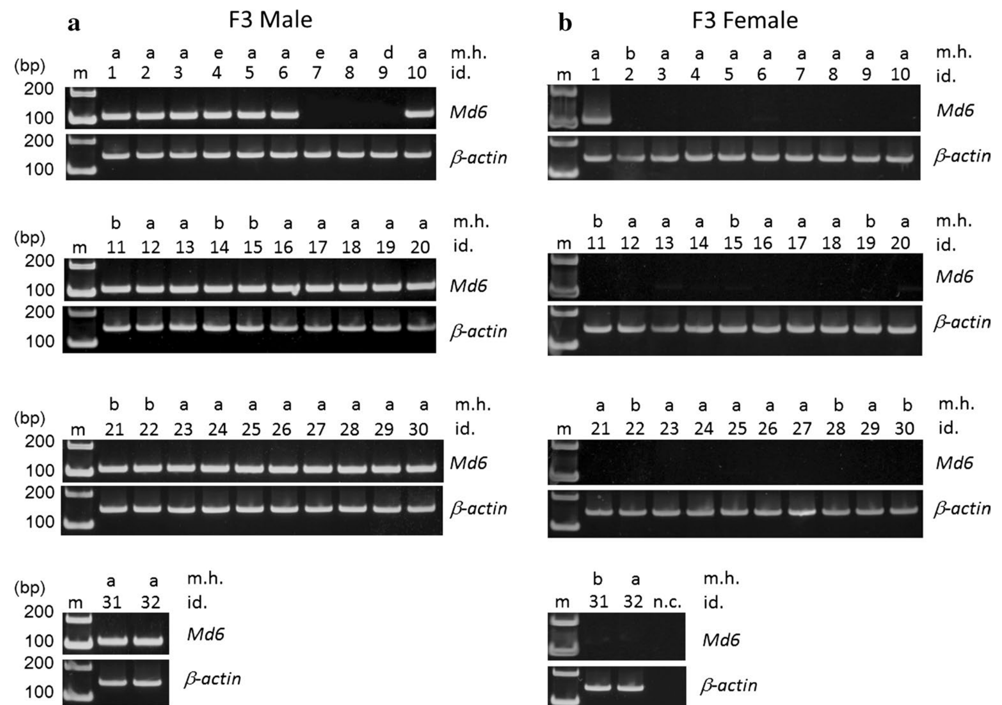

Fig. 3 Genotyping PCR and mitochondrial haplotype analysis of F3 Pacific bluefin tuna, **a** males and **b** females. m.h., mitochondrial haplotype and id., individual *numbers* are indicated at the *top* of the electrophoresis images. *m* molecular size marker and *n.c.* negative control without template DNA. PCR products for *Md6* (approximately 120 bp) and the internal control *β-actin* (180 bp) were shown

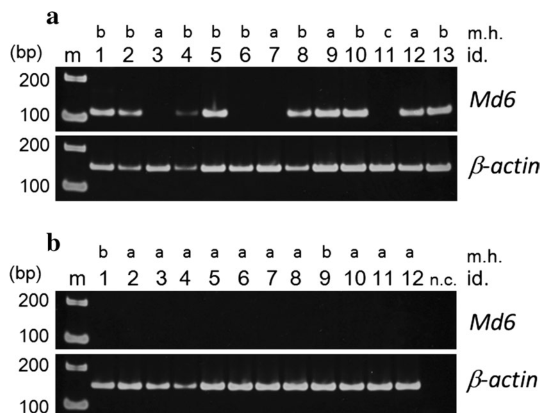

Fig. 4 Genotyping PCR and mitochondrial haplotype analysis of F2 Pacific bluefin tuna **a** males and **b** females; *m.h.* mitochondrial haplotype and *id.* individual *numbers* are indicated at the *top* of the electrophoresis images. *m* molecular size marker, *n.c.* negative control without template DNA. PCR products for *Md6* and the internal control *β-actin* are shown

Results suggested that cultured PBF stock may have a male heterozygous sex chromosome system, as indicated by the male-dominant marker of *Md6* detected in F2 and F3 males (Figs. 3, 4). However, this was not seen frequently, one female progeny carried *Md6*, which may be the result of meiotic recombination between *Md6* and a null allele, with a frequency of approximately 3.4 % (1 of 29). These results suggest that *Md6* is linked to a male sex chromosome in cultured PBF but not in wild fish (Table 1), and that *Md6* is not directly associated with sex determination.

Parentage allocation of the cultured fishes

To further confirm the *Md6* segregation of F2 to F3 males, parentage allocation was conducted [15–18]. In mitochondrial haplotype a (Figs. 3, 4), the best candidate paternal fish was F2 male id. 2 (Table 2, supplemental Table 2), the second was F2 male id. 13, and the third candidate was F2 male id. 12, all of which were were *Md6*-positive (Fig. 4). Interestingly, F2 male id. 3 and 11 were *Md6*-negative, with a 0 score of less possibility to be paternal fish of F3. F2 male id. 6 and 7 were also *Md6*-negative, and there was little possibility that the F2 male id. 6 was a paternal fish. This was implied in the result showing that only two F2 (male id. 5 and 6) among all F2 fishes had *Ttho-1* allele 180 bp, and one of 48 F3 haplotype a (id. Female 14) had *Ttho-1* allele 180 bp (supplemental Table 2). These results indicate that more than one male were attendant in spawning. The best candidate maternal fish haplotype a was suggested as a F2 female id. 2 (Table 2).

In haplotype b, the top three candidate parental fishes were F2 male id. 1, 8, and 10 of the *Md6*-positive, with similar score values. Among the four F2 *Md6*-negative males, only F2 male id. 6 had little possibility of participation in the spawning event (Table 3). In maternal fish of haplotype b, we think it is likely that the fish other than the F2 female id. 1 and 9 contributed significantly to the spawning (Table 3). The *Ttho-8* 295 bp allele was observed in 9 of 13 F3 haplotype b fishes; however, none of the F2 male and F2 haplotype b females had the *Ttho-8* 295 bp (supplemental Table 3).

Table 1 Summary of the *Md6* genotyping results of aquacultured and wild PBF

	Male				Female			
	Positive	Negative	Total (n)	Positive (total %)	Positive	Negative	Total (n)	Positive (total %)
Fish group								
2002 F2 cultured	9	4	13	54.5	0	12	12	0
2007 F3 cultured	29	3	32	90.6	1	31	32	3.1
2004 Wild	0	4	4	0	2	3	5	40.0
2007 Wild	2	18	20	10.0	6	14	20	30.0

Table 2 Summary of the parentage allocation of cultured PBF mitochondrial haplotype a using the exclusion method

Haplotype a

F2 male id.	1	2	3	4	5	6	7	8	9	10	11	12	13
Total score	3.62	10.91	0	2.37	2.65	3.45	4.44	4.98	3.02	1.17	0	4.99	5.75

F2 female id.	2	3	4	5	6	7	8	10	11	12	Others
Total score	1.77	15.96	3.91	4.54	4.36	3.82	3.15	3.82	3.99	0.62	2

Total score indicates sum of the possibilities.

Table 3 Summary of the parentage allocation of cultured PBF mitochondrial haplotype b using the exclusion method

Haplotype b

F2 male id.	1	2	3	4	5	6	7	8	9	10	11	12	13
Total score	2.15	0.83	0	0	1.41	0.25	0	2.32	1.50	2.00	0	1.50	0.99

F2 female id.	1	9	Others
Total score	2.00	5.00	6.00

Total score indicates the sum of the possibilities.

Discussion

We would like to note the characteristics of the *Md6*. We first performed sequence analysis of the male characteristic fragment obtained with AFLP using a standard method of T-A cloning, followed by dye terminator labeling. However, we could not obtain clear electropherograms because those generated from the candidate fragment became complex at position 173, corresponding to the 6-bp deletion (Fig. 2). With the use of pyrosequencing, the nucleotide length of *Md6* was identified as 408 bp, whereas the male characteristic fragment amplified by AFLP was observed at 440 bp (Fig. 1) [11], using non-denaturing gel electrophoresis. This discrepancy was likely the result of the reduced electromobility of the *Md6* fragment caused by a heteroduplex formation with redundant sequences, as their sequences were nearly identical to the *Md6* without 6-bp deletion (Fig. 2), and few substitutions and deletions. The heteroduplex may be formed during the later cycles of AFLP secondary selective PCR amplification. The decreased electromobility of the heteroduplex fragment has been reportedly utilized for the rapid detection of nucleotide substitution and/or small deletions in known sequences [22, 23]. The heteroduplex formation of *Md6* and redundant sequences was supported by the read count of the sequences generated using high-throughput sequencing. In the male sequences, the five most frequent sequences comprised 2,852 reads, among which *Md6* comprised 419, resulting in an *Md6* total read ratio of 14.6 %. This read count ratio was nearly the same even when the 30 most frequent sequences in males were counted, where *Md6* comprised 1,023 reads among a total of 7,932 reads, resulting in a ratio of 12.8 % (Table S1). The abundance ratio of the male characteristic fragment of 440 bp relative to the redundant 420 bp fragment observed in automatic non-denature electrophoresis was estimated at 11 % in our previous study [11], and this was nearly identical to above-mentioned ratios. In addition, in this study, we analyzed AFLP products that were amplified with the FAM-labeled *Mse*I+CAT primer using an ABI Prism 3130 analyzer, and the male characteristic fragment was observed at 408 bp in male individuals (data not shown). These results indicate that the major feature of the nucleotide sequence of the male characteristic fragment was 6-bp deletion polymorphism compared to the redundant sequences.

The nucleotide sequence of *Md6* and redundant sequences were nearly identical (Fig. 2, S1). Such a feature of sex chromosome-linked sequence was reported in the

medaka fish *O. hubbsi* W chromosome repetitive heterochromatic region [24]. In addition, we performed a BLAST search of *Md6* in genomic sequencing of PBF [20], with the results indicating the highest score for the *BADN01109032* sequence, which was 2,210 bp in length and shared 90 % sequence identity with *Md6*. The longest contig reported for PBF was 79,504 bp, with an average of 3,813 bp [20]; *BADN01109032* may be restricted for assembly due to the repetitive region.

In determining the heterogenesis of *Md6* (Figs. 3, 4), we found that *Md6* was likely enriched after successive generations during aquaculture. In F2 males, 9 of 13 (69 %) were *Md6*-positive, and it was appeared that most of the sperm was provided by *Md6*-positive F2 males, which was supported by the parental allocation study (Tables 2, 3). Some candidate paternal F2 fish were *Md6*-positive, while *Md6*-negative F2 males were less possible than *Md6*-positive. This can be explained by the spawning ecology of PBF, which is reported as multiple males participating in the event ([12] and our observations), and the sperm supply in the event was not equal. This could mean that males carrying *Md6* were culture-selected. Details will be determined in future studies, but *Md6* males likely have advantages in surviving and growing or spawning under aquaculture conditions.

A molecular marker of *Md6* was expected to be effective for the F4 male marker, as we first obtained F4 fingerlings in 2012, and 53 % ($n = 81$) were *Md6*-positive, although gonad sex phenotypes were not known at that time (data not shown). However, we should note that *Md6* genotyping included 6.2 % (4/64) error in F3; three of 32 males were negative, and one of 32 females was positive (Fig. 3).

Genetic sex markers are very important for the genetic management and reproduction in aquaculture fish populations. In the last decade, identification of sex-linked DNA markers were enhanced in several aquaculture fish species. For example, a linkage map of the rainbow trout *Oncorhynchus myskiss* was constructed primarily using microsatellites, and its sex-linked marker was identified [25]. Half-smooth tongue sole *Cynoglossus semilaevis* females grow faster than males, and therefore, female monosex production is useful. The fish female DNA marker was identified using AFLP analysis that showed the fish has a female heterozygous sex chromosome [26]. Yellowtail *Seriola quinqueradiata* is also a very important commercial fish in Japan. Microsatellite linkage analysis was conducted in the fish, and a W chromosome-specific DNA marker was identified [27]. Recently, in addition to sex markers, sex-determining genes have been identified, and it was suggested that these genes are involved in the TGF-β signaling pathway [28]. It is uncertain whether known sex-determining genes or genes associated with TGF-β signaling are close to *Md6*; therefore, our next step is to find conserved genes around *Md6* as molecular bait. If we can find coding genes linked to *Md6*, well-studied fish genomic data will be quite useful for comparative study, such as in the expectation of synteny. This may contribute to the identification of much more concrete DNA markers for sex genotyping of tuna, and also may enhance PBF broodstock management.

It was worth noting the sex-determining locus of our PBF. We performed *Md6* genotyping in F2 and F3 (Figs. 3, 4). In F3 fish, 29 of 32 males were *Md6*-positive, and 1 of 32 females was positive. If we infer that the ratio of the sperm provided from *Md6*-positive and null males was roughly 29:3 when F2 spawned, the ratio may be same in F3 females. We could then that see one F3 female was *Md6*-positive, and therefore the recombination rate would be 1 in 29 (3.4 %) in F2 males. We would then assume that the putative sex-determining locus or gene would be roughly a 3.4 % recombination rate distant from *Md6*.

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