

Molecular Identification of an “Abnormal Tuna” Caught in the Taiwan Strait off Southwestern Taiwan

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Abstract

A female tuna of odd appearance, with the size of a Pacific bluefin tuna (*Thunnus orientalis* Temminck and Schlegel, 1844), anal fin count of bigeye tuna (*Thunnus obesus* Lowe, 1839), pectoral fins of yellowfin tuna (*Thunnus albacares* Bonnaterre, 1788), caught in waters west of the island of Xiaoliuqiu, Pingtung, Taiwan, received considerable media attention after its landing at Tung kang fishing port on 14 December 2020. This tuna has now been identified by molecular systematic means. Fishermen and fish merchants suggested it was a hybrid form, unique in living memory; however, it had matured ovaries and developing ova. To confirm whether this fish was a hybrid, we determined its parental species by analysing the cytochrome *b* gene (*cyt b*) and cytochrome *c* oxidase subunit 1 (*COI*) sequences of the mitochondrial DNA for maternal inheritance, and the internal transcribed spacer 1 (*ITS1*) gene sequence from the nuclear DNA to confirm both parents' lineages. Genomic DNA was isolated from fast-skeletal muscle, and primers were designed based on the known sequences of conserved regions among tunas. According to *cyt b* and *COI*, the mother of the peculiar tuna was a Pacific bluefin tuna (*T. orientalis*), and the *ITS1* sequence showed that both parents were of this species. We therefore conclude that despite the mixed morphological appearance, this abnormal tuna was a Pacific bluefin tuna, not a hybrid.

Keywords: tuna fishery, putative hybrid, mitochondrial DNA, nuclear DNA, parental inheritance markers

Introduction

The evolution of species is an ongoing process, and events such as mutation or hybridisation may trigger the evolution of a new species (Abbott et al., 2013; Pfennig et al., 2016). Fishermen caught a peculiar tuna off southwestern Taiwan on 13 December 2020 in the Taiwan Strait west of the island of Xiaoliuqiu (Fig. 1) and landed it the following day at Tung kang fishing port. This tuna displayed a mixture of characteristics of Pacific bluefin tuna (*Thunnus orientalis* Temminck and Schlegel, 1844; hereafter referred to as bluefin tuna), bigeye tuna (*Thunnus obesus* Lowe, 1839), and yellowfin tuna (*Thunnus albacares* Bonnaterre, 1788) (Fig. 2). This tuna, with its peculiar combination of physical characteristics, became a topic of local and national news and was widely reported in the Taiwan media on

16 December 2020, including on YouTube (<https://www.youtube.com/watch?v=vSTbzeD0wtw&t=50s>; <https://www.youtube.com/watch?v=l6UnznyMRuQ&t=17s>).

Many fishermen, freezing-plant operators, and tuna vendors stated that in the past 30 years they had never seen such an unusual fish in the tuna fishery. Fisheries-related companies speculated that the fish may have been the result of hybridisation between a bluefin tuna and either a bigeye or a yellowfin tuna. Natural hybrids often occur in fish, both marine (Yokogawa and Urayama, 2000; Sueyoshi et al., 2009; Takahashi et al., 2017; Tatsuno et al., 2019) and freshwater (Elder et al., 1971; DeMarais et al., 1992; D'Amato et al., 2007). Many artificially hybridised species of farmed fish have also been recorded, although far more often for freshwater fish than marine fish (Bartley et al., 2000; Romana-Eguia et al.,

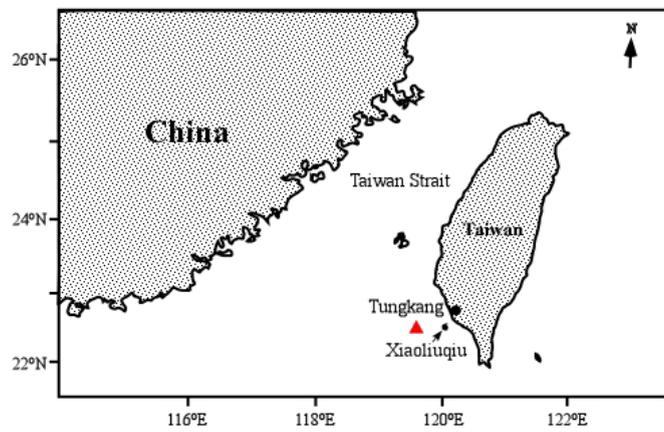


Fig. 1. Capture location map of the abnormal tuna. The red triangle (▲) indicates the sampling site of the abnormal tuna (119.85E, 22.35N).

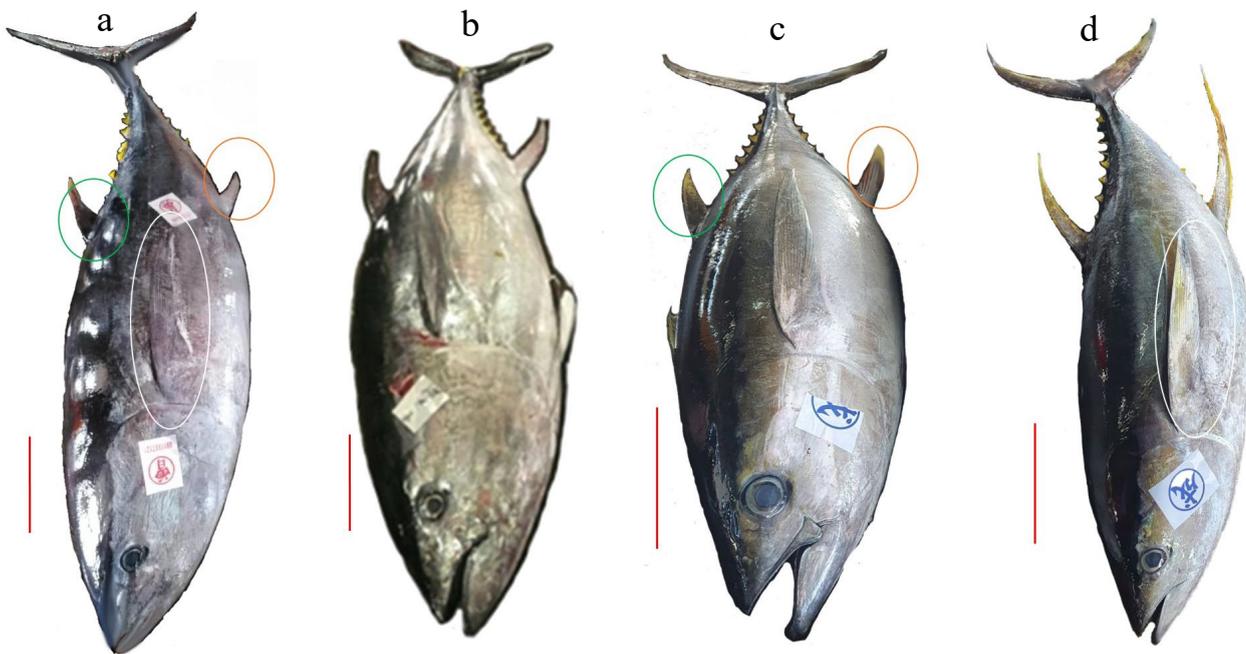


Fig. 2. The abnormal tuna (a), Pacific bluefin tuna (b), bigeye tuna (c), yellowfin tuna (d). The abnormal tuna and other tuna were landed in Tungkuang Fishing Port. The color circles indicated the same parts. The photos (taken on 14 December 2020) were provided by Mr. Han-Chou Lin, Hua-Ciao Fishing Market, Tungkuang, Taiwan. Scale bars = 30 cm.

2004; Wang et al., 2012; Phadphon et al., 2019; Rahman et al., 2019). However, there are yet no reports of natural hybridisation of tuna. The news reports from Tungkuang fishing port inspired us to try to collect the abnormal and putatively hybrid tuna to determine whether its unique appearance was indeed due to hybridisation and not, for example, to the effects of a single-locus mutation.

Yellowfin tuna are mainly found in tropical regions (Song et al., 2008), while bigeye and bluefin tuna are also distributed in temperate regions (Goujon and Majkowski, 2000; Kitagawa et al., 2006). Furthermore, yellowfin tuna mainly occurs in shallow waters, while bigeye and bluefin tuna are distributed in the deeper parts of the ocean (Goujon and Majkowski, 2000).

According to the official website of the Tungkuang Fishermen's Association (<https://www.tkfisher.org.tw>), bluefin tuna are mainly caught from April to July whereas bigeye and yellowfin tuna are caught throughout the year. Their similar spawning behaviour (Goujon and Majkowski, 2000) suggests that the hybridisation of bluefin and bigeye tuna is more likely than that of bluefin and yellowfin tuna or of bigeye and yellowfin tuna. Bluefin/bigeye hybridisation thus became our null hypothesis, which led to the additional question of which parent fish belonged to which species.

Identification of the maternal and paternal lineages of hybrids often involves the analysis of two different sorts of DNA that are indicative of maternal and

paternal inheritance respectively. For example, Ichimura et al. (2011) used mitochondrial DNA (mtDNA: ATPase 6 and NADH3) and nuclear DNA (nDNA: *ITS1*, *ITS2*, and Aromatase B-1) to identify several specimens of so-called "sakemasu" as hybrids of chum salmon *Oncorhynchus keta* (Walbaum, 1792) fathers and pink salmon *O. gorbuscha* (Walbaum, 1792) mothers. In addition, Kijewska et al. (2009) used mtDNA (cytochrome *b* and D-loop) and nDNA (ribosomal marker ITS and parathyroid hormone-related protein gene) to identify a hybrid flatfish, *Platichthys flesus* (Linnaeus, 1758) × *Pleuronectes platessa* Linnaeus, 1758. For fish in general, the most commonly used mtDNA markers for determining maternal inheritance are 16S rRNA (Inoue et al., 2001; Hashimoto et al., 2010; Cawthorn et al., 2012; Porto-Foresti et al., 2013), the cytochrome *b* gene (*cyt b*) (Bartlett and Davidson, 1991; Chow and Kishino, 1995; Aboim et al., 2010), cytochrome *c* oxidase subunit 1 (*COI*) (Paine et al., 2007), and ND-1 (Politov et al., 2000). For nuclear DNA, both maternal and paternal inheritance are commonly analysed by sequencing internal transcribed spacer 1 (*ITS1*) (Chow and Kishino, 1995; Chow et al., 2006) and the genes for such proteins as α -tropomyosin (Hashimoto et al., 2010), creatine kinase (Politov et al., 2000), and rhodopsin (Rehbein, 2013). Half of the nuclear DNA comes from the paternal line and the other half comes from the maternal line, so by designing paternal- or maternal-specific primers, both parents' genes can be identified.

To address these points, we tried to determine the maternal species of the abnormal tuna by molecular biological means based on the principle of maternal inheritance. The *cyt b*, *COI* and 16S rRNA genes of the mitochondrial genome were selected as suitable target sequences. In addition, the *ITS1* sequences of the nuclear genome were used as markers to confirm the both the maternal and paternal identity for this tuna.

Materials and Methods

Ethical approval

No live animals were used in this study. Therefore, no Institutional Animal Care and Use Committee (IACUC) approval was required.

Sampling

The peculiar tuna specimen was found at the Hua-Ciao Fishing Market, Tungkang City, Pingtung County, Taiwan, on 17 December 2020. According to Mr. Han-Chou Lin (Director General of the Tungkang Fishermen's Association) it had been caught by the fishing boat "Manjisheng" captained by Mr. Kun-Shan Hong on 13 December 2020 at 22.35°N, 119.85°E (west of the island of Xiaoliuqi in the Taiwan Strait) by longline fishing with moonfish (*Mene maculata* Bloch and Schneider, 1801) as bait. According to data from the Central Meteorological Bureau of Taiwan, the water temperature at the site was 20.0–20.4 °C. December is not the usual season

for landing bluefin tuna in Tungkang, so the raw meat (sashimi) and head of this peculiar tuna quickly sold out at an auction price equivalent to about USD30 kg⁻¹ and by the time we arrived, only the vertebrae and adhering muscles were left. These parts of the fish were placed in a styrofoam box with ice, brought back to our laboratory in Tainan, and stored there at -20 °C until use. The fish had been photographed before being processed for sale (Fig. 2A). Photos and other biological information were obtained from Mr. Han-Chou Lin. Authentic specimens of bluefin, bigeye, and yellowfin tuna that had been identified by local professional tuna-trading people were obtained at the same fish market and used as reference fish. The reference fish had identified species by *COI* sequence and had submitted to nucleic acid sequence data banks (DDBJ/EMBL/GenBank), they were identified as bluefin (DDBJ/EMBL/GenBank accession numbers: OK999971, OL257871, and OL257849, bigeye (OLO14470 and OLO22303), and yellowfin (OLO29561) tunas.

Genomic DNA extraction

Samples of skeletal muscle weighing 25 mg were excised with scissors from the dorsal part of the peculiar tuna and each reference specimen. The Qlamp DNA Mini Kit Nucleic Acid Purification Kit (QIAGEN, Germany) was used to obtain purified genomic DNA.

Primers and PCR

The primers used for gene amplification (Table 1) were designed based on the sequences of the genes encoding *cyt b* (primers designed by us), *COI* (Ward et al., 2005), 16S rRNA (Palumbi et al., 1991; Inoue et al., 2001), and *ITS1* (Chow and Kishino, 1995) of bluefin, yellowfin, and bigeye tuna (Table 1). Two primer pairs of *cyt b* were used, *Cytb-F* and *Cytb-R*. Besides the primers *ITS1F* and *ITS1R* designed by Chow and Kishino (1995), three primers (*obF30*, *obR391*, and *obR424*) designed by us specifically for bigeye tuna were also used in order to more accurately confirm whether the bigeye tuna gene was contained in the abnormal tuna's genomic DNA (Table 1). The *cyt b*, *COI*, 16S rRNA, and *ITS1* genes were amplified by PCR using a MyCycler™ Thermal Cycler System (Bio-Rad, USA) and the following quantities and concentrations of substrates and reagents: 2 μ L of 10 × Ex Taq Buffer (Mg²⁺ plus), 2 μ L of 2.5 mM dNTP mixture, 0.2 μ L of Ex Taq (5 U. μ L⁻¹; Takara, Japan), 2 μ L of template DNA, 2 μ L of each primer (1 μ M) and 9.8 μ L of Milli-Q water to give a total reaction volume of 20 μ L (Ishizaki et al., 2006). PCR amplification consisted of initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 2 min, and a final extension at 72 °C for 5 min. The PCR product was subjected to electrophoresis using 1 % agar (VWR Funding Inc., USA) and visualised with SYBR Green (HealthView Nucleic Acid Stain, Genomics, Taiwan). The marker used was DM2300 ExcelBand (SMOBIO Technology, Inc., Taiwan).

Table 1. List of primer pairs and PCR annealing temperatures used to amplify the genes encoding *cyt b*, *COI*, *ITS1*, and 16S rRNA.

Name	Sequence (5'-3')	Annealing temperature (°C)
Mitochondrial genes		
<i>cyt b</i> (primers designed by us)		
CytbF	GAATCAGCCTTCGCCTCAGT	50
CytbR	GATGGAGGCTAGGAGGGCTA	
<i>COI</i> (Ward et al., 2005)		
Fish F1	TCAACCAACCACAAAGACATTGGCAC	54
Fish R1	TAGACTTCTGGGTGGCCAAAGAATCA	
16S rRNA (Palumbi et al. 1991; Inoue et al. 2001)		
16SAR	CGCCTGTTTATCAAAAACAT	52
16SBR	CCGGTCTGAACTCAGATCACGT	
Nuclear genes		
<i>ITS1</i> (Chow and Kishino, 1995)		
ITS1F	GTCGTAACAAGGTTTCCGTA	62
ITS1R	GTGCCGAGTGATCCACCGCT	
<i>obesus ITS1</i> (primers designed by us)		
obF30	GCGCGACAAGACGCCTCC	62
obR391	TCCGAGTGTCCGACCGGGC	
obR424	AAGGCTCCGCTTCCGCGC	

Sequencing

The PCR products were sent to a biotech company, Genomics (New Taipei City, Taiwan), and purified and sequenced. Bidirectional sequencing was carried out using the forward and reverse primers mentioned above. Sequenced chromatograms were checked using CHROMAS 2.23 (Technelysium, Australia). Finally, the forward and reverse sequence fragments were assembled using BioEdit 7.2 (<https://bioedit.software.informer.com/7.2/>) and Multiple Sequence Alignment (Clustal Omega - GenomeNet).

Molecular systematic analysis

Comparisons of the edited and aligned *cyt b*, *COI*, 16S rRNA, and *ITS1* sequences of the abnormal tuna and the three other tuna species (bluefin, bigeye, and yellowfin tuna) were performed using Molecular Evolutionary Genetics Analysis ver. 11 (MEGA 11) software (Tamura et al., 2021). Comparative sequence data were obtained from the National Center for Biotechnical Information (NCBI): *Cyt b* sequences for *Thunnus orientalis* (MG017672), *T. obesus* (EU224036), and *T. albacares* (MG017682); *COI* sequences for *T. orientalis* (OL257849), *T. obesus* (OL014470), and *T. albacares* (OL029561); 16S rRNA sequences for *T. orientalis* (JN097816), *T. obesus* (GQ461734), and *T. albacares* (GU946661); and *ITS1* sequences for *T. orientalis* (EU708983), *T. obesus* (AB212025), and *T. albacares* (EU708982). Bootstrap majority consensus values for 1,000 replicates were calculated (Felsenstein, 1985) and indicated as percentages at each branch node.

Results

External features

The peculiar tuna's overall appearance (Fig. 2A) could only be judged based on news videos and photographs.

It was estimated to be about 186 cm long (estimate total length) and weighed 133 kg. The pectoral fins were very long, even reaching the second dorsal fin, thus being unlike those of bluefin tuna (Fig. 2B) but closer in the form to those of yellowfin tuna (Fig. 2D). While the eyes and body size resembled those of bluefin tuna (Fig. 2B), the overall appearance and the pectoral fins resembled those of yellowfin tuna. The anal fin looked like that of a bigeye tuna (Fig. 2C).

Internal features

According to Mr. Han-Chou Lin of the Tungkang Fishermen's Association, the fish was female, with ovaries and immature oocytes. He reported that the 'harakami' (the part near the head on the belly side) was similar to that of bluefin tuna (Fig. 3A), with a thick fat layer and rather elastic flesh. The 'ootoro' (the meat of the 'harakami' area) was bright pink with vibrant white lines, thus similar to that of bluefin tuna; however, the dorsal flesh 'akami' was similar to that of yellowfin tuna (Fig. 3B).

Mitochondrial genes

Cyt b gene

The nucleotide sequence we obtained for *cyt b* (DDBJ/EMBL/GenBank accession No. MW969644) consisted of 691 bases encoding 230 inferred amino acid residues (Table 2). The latter were identical with those of bluefin tuna, and the nucleotide identity between the peculiar tuna and bluefin tuna was 99.85 % (Table 2). Multiple sequence alignment using Clustal Omega showed at least 19 nucleotide positions that differ from the other two species (Fig. 4).

COI gene

The *COI* gene was also successfully cloned, giving 684

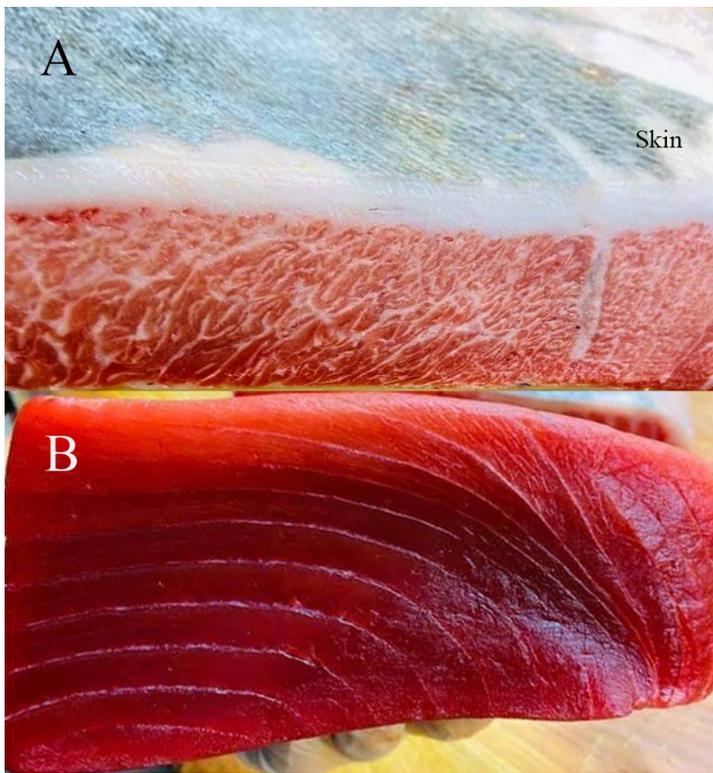


Fig. 3. Appearance of the abnormal tuna muscle. (A) ventral (belly) muscles with marbling similar to that of bluefin tuna. (B) cross section of the dorsal red muscle (akami). Photos provided by Zeng Xian Sashimi Bluefin Tuna Specialty Store, Tungkuang, Taiwan, 14 December 2020.

Table 2. The peculiar tuna's nucleotide and amino acid sequence identities with three reference species.

Gene	Bluefin tuna	Bigeye tuna	Yellowfin tuna
Mitochondrial genes			
cyt b (691 bp)			
Matched nucleotides	690/691	663/691	664/691
Identity (%)	99.85	95.94	96.09
GenBank references	MG017672	EU224036	MG017682
CYT b (230 inferred residues)			
Matched amino acids	230/230	230/230	230/230
Identity (%)	100.00	100.00	100.00
GenBank references	AXE42972	ABW97015	AXE42982
<i>COI</i> (625 bp)			
Matched nucleotides	625/625	614/625	616/625
Identity (%)	100.00	98.24	98.56
GenBank references	OL257849	OL014470	OL029561
16S rRNA (561 bp)			
Matched nucleotides	559/561	560/561	560/561
Identity (%)	99.64	99.82	99.82
GenBank references	JN097816	GQ461734	GU946661
Nuclear genes			
<i>ITS1</i> (626 bp)			
Matched nucleotides	622/626	567/626	535/626
Identity (%)	99.36	90.57	85.46
GenBank references	EU708983	AB212025	EU708982
obesus <i>ITS1</i> (370 bp)			
Matched nucleotides (ab1*)	367/370	337/370	334/370
Identity (%)	99.19	91.08	90.27
Matched nucleotides (ab2**)	369/370	337/370	334/370
Identity (%)	99.73	91.08	90.27
GenBank references	EU708983	AB212025	EU708982

*abnormal 1, **abnormal 2

bp in total (DDBJ/EMBL/GenBank accession numbers OM847398). A comparison of the *COI* sequences of the abnormal tuna and the other three tuna species (Fig. 5)

shows 100 % similarity of the former with bluefin tuna, 98.24 % with bigeye tuna, and 98.56 % with yellowfin tuna (Table 2).

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Abnormal tuna CGCCTCAGTAGCCACATTGCGGAGATGCAACTTCGGTTGACTCATCCGGAACCTCCACGCAACGGGGCCTCTTTCTTTTACTGCATCTACTTC 100
T. orientalis ..... 100
T. obesus .....T..... 100
T. albacares ..... 100

Abnormal tuna CACATCGGCCGAGGCCTTTACTAGGGCTTTACCTGTACAAAGAAACATGAAACATCGGAGTAGTGCTCCTACTTCTAGTCATGATGACCGCCTTCGTTG 200
T. orientalis .....T..... 200
T. obesus .....A.....A.....A..A.....T..... 200
T. albacares .....A.....A.....G.....A.....C.....T..... 200

Abnormal tuna GCTACGTCCTTCCCTGAGGACAAATGTCCTTCTGAGGAGTACCGTCATTACTAACTCCTATCCGCGAGTCCCATATGTGGAACTACCCCTCGTTGAATG 300
T. orientalis ..... 300
T. obesus .....G...T.....C..T.....C.....T..... 300
T. albacares .....T.....C..T.....T..... 300

Abnormal tuna AATCTGAGGGGGCTTTTCAGTAGCAATGCTACCCTTACCCGATCTTCGCACTTCCACTTCTATCCCATTCGTCATCGCAGCCATGACAATTCTTCAC 400
T. orientalis ..... 400
T. obesus .....C...C.....T..... 400
T. albacares .....A...C.....C.....T..... 400

Abnormal tuna CTCTTTTCCCTCAGCAACAGGTTCAATAATCCAATCGGATTAAACTCAAATCGAGATAAAATCTCATTCCACCCATACTTCTCTTACAAAGACCTCC 500
T. orientalis ..... 500
T. obesus ..... 500
T. albacares .....C..... 500

Abnormal tuna TTGGTTTCGTCATCCTGCTAGTGGCACTTGCCTCTCTAGCACTATTCTCCCTAACTCCTGGGAGACCCAGACAACCTTACCCTGCTAACCCGATGGT 600
T. orientalis .....A.... 600
T. obesus .....T.....A.....C.....T.....A.....C.....C..... 600
T. albacares .....A.....C.....C.....A.....C.....T..... 600

Abnormal tuna CACCCACCTCACATTAACCTGAATGATACTTCTTATTGCTTACGCAATTCTTCGATCTATTCCAAACAACCTAGGAGGAGTACTAGCC 691
T. orientalis ..... 691
T. obesus T.....T.....A.....C.....G..... 691
T. albacares T.....T.....A.....G..C..... 691

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Fig. 4. Alignment of the *cyt b* nucleotide sequences obtained from the abnormal (DDBJ/EMBL/GenBank accession number MW969644), Pacific bluefin (*Thunnus orientalis*, MG017672), bigeye (*T. obesus*, EU224036), and yellowfin (*T. albacares*, MG017682) tunas.

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Abnormal tuna TGAGCTGGAATAGTTGGCACGGCCTTAAGCTTGCTCATCCGAGCTGAACTAAGCCAACCAGGTGCCCTTCTTGGGGACGACCAGATCTACAATGTAATCG 100
T. orientalis ..... 100
T. obesus ..... 100
T. albacares ..... 100

Abnormal tuna TTACGGCCCATGCCTTCGTAATGATTTTCTTTATAGTAATACCAATTATGATTGGAGGATTTGGAACTGACTTATTCTCTAATGATCGGAGCCCCCGA 200
T. orientalis ..... 200
T. obesus ..... 200
T. albacares ..... 200

Abnormal tuna CATGGCATTCCACGAATGAACAACATGAGCTTCTGACTCCTTCCCTCCTTTCTCTCTGCTCCTAGCTTCTTCAGGAGTTGAGGCTGGGGCCGGAACC 300
T. orientalis ..... 300
T. obesus .....C.....T.....A..... 300
T. albacares .....C.....A..... 300

Abnormal tuna GGTGGAACAGTCTACCCTCCCTTCCGCGCAACCTAGCCACGCGAGGGCATCAGTTGACCTAACTATTTTCTCACTTCACTTAGCAGGGGTTTCTCAA 400
T. orientalis ..... 400
T. obesus .....G.....G.....G..... 400
T. albacares .....A.....G..... 400

Abnormal tuna TTCTTGGGGCAATTAACCTCATCACAAATTATCAATATGAAACCTGCAGCCATCTCTCAATATCAAACACCCTGTTTGTATGAGCTGTACTAATTAC 500
T. orientalis ..... 500
T. obesus .....T..T...G..... 500
T. albacares .....T..T...G..... 500

Abnormal tuna AGCTGTTCTTCTTACTTTCCCTTCCAGTCCCTTCCGCTGGTATTACAATGCTCCTTACAGACCAGAACTAAATACAACCTTCTTCGACCTGCAGGA 600
T. orientalis ..... 600
T. obesus .....C..... 600
T. albacares .....C..... 600

Abnormal tuna GGGGGAGACCCAATCCTTTACCAGC 625
T. orientalis ..... 625
T. obesus .....A. 625
T. albacares .....A. 625

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Fig. 5. Alignment of the *COI* nucleotide sequences obtained from the abnormal tuna (DDBJ/EMBL/GenBank accession number OM847398) and Pacific bluefin (*Thunnus orientalis*, OL257849), bigeye (*T. obesus*, OL014470), and yellowfin (*T. albacares*, OL029561) tunas.

16S rRNA gene

A total of 561 bp of the 16S rRNA gene was obtained (DDBJ/EMBL/GenBank accession number MW960054).

However, the 16S rRNA sequences did not discriminate among the species of bluefin, bigeye, and yellowfin tuna, because all the tested fish were almost identical in this respect (Table 2).



Nuclear gene

ITS1

The *ITS1* gene, as part of the nDNA, exists in the nucleus in two copies originating respectively in the paternal and maternal lines. Using two primers, ITS1F and ITS1R (Chow and Kishino, 1995), we obtained two slightly different 626 bp sequences (DDBJ/EMBL/GenBank accession number MW960117), differing at two sites, that were both most similar to that of bluefin tuna, 99.36 % or 99.73 % identical respectively (Table 2), whereas at least 17 positions differed between these fish and the sequences of bigeye and yellowfin tuna (Fig. 6). The “abnormal tuna 2” sequence was only 91.08 % identical to that of bigeye tuna (Table 2). Similar results were obtained using our

own bigeye-tuna-specific primers for *ITS1* (obF30, obR391, and obR424). Six clones of *ITS1* were obtained by PCR amplification by primers obF30-obR391, obF30-obR424, and ITS1F-obR391, with an intersection sequence of 370 bp (Fig. 7). The sequences obtained from the abnormal tuna (DDBJ/EMBL/GenBank accession number OM851967) were 99.73 % identical to the corresponding sequences of bluefin tuna, but only a 91.08 % match to those of bigeye tuna (Fig. 7; Table 2).

Phylogenetic analysis

The results of a phylogenetic analysis using the two markers mtDNA (*cyt b*) and nDNA (*ITS1*) are shown as phylogenetic trees (Figs. 8, 9). The abnormal tuna shows a close relationship with bluefin tuna in both these analyses.

Abnormal tuna	CCGGTTTGCCCGCCCTGTCGCGAGGGGCGCGCAACA-GACGCCCTCCGACAGCTGAGGGCCAGAGGGTAGGTCTCTTCCCCCCCCGGGTCCCGTCGCG	99
T. orientalisG..T.....	99
T. obesusC.....G..A.....G....CG.....-G.....	99
T. albacaresC.....G..A.....G....CG.....G.....	100
Abnormal tuna	GTCGTCTCCCGAGGACTTCCCGGGCGCGCGCGCGGCG---GTGGTCGTTTCCCTCCCG-AAGGCCCGAGGCGCGCGC---GGGCGCTTTTCTTCCCGAG	191
T. orientalisC.....	191
T. obesusCGG-C.....-C.....TGTC.....C.....	197
T. albacaresCGG.C.....TT.C.....GTGG.....C.....	200
Abnormal tuna	ACGC---CCCGCGCAAAACACCTCTCCTGGTCAGGACCTCGTCCCGACCGGGCGGGCCCT-CCCCACTTCTCCCCCCCCCTGGGCGACACGCCTCGCGACC	286
T. orientalisT.....	286
T. obesusTCCC.....T.....T.....GT.....	297
T. albacares	T...TCCC.....T.....GT.....-.....AC...	299
Abnormal tuna	CCGGCCAGCCTAAGCTCCCGGGGCTGACGGAGCGG-CGGGTCCGACGTCGGGGCGCCCTCGCGGGGGTTCGGGGACCCGTCGGGTGCACCGGGGCTGGG	385
T. orientalis	385
T. obesusT...G.....G.....-.....C..T	396
T. albacaresT...G.....G.....-.....C..T	398
Abnormal tuna	CCGACACTCCGGAACCTTAACCC-AAAGCGCGCAGCGGAGGC-TTCGCCCCCGCGCACGCGCGCGCCCCGGGTACCCAACTCTCCCCCTCCTTCG	483
T. orientalis	483
T. obesusA.....C.....	495
T. albacaresC.....A.....C.....	498
Abnormal tuna	GAGGGAGGAGGGGGTTCAATGTCTCCCCACCCCGCCTCGGGGGGTTCCGAGCGCCCGGGGGTCTGTCTCTTCCCTTTCCAAACCCGACTTGTCTC	583
T. orientalis	583
T. obesusG.....C.....	595
T. albacaresG.....C.....	598
Abnormal tuna	TGACGGTGGCAACCTCTGTGCGGTGTA AAAACCGACAAAAA	626
T. orientalisT.....	626
T. obesusT.....	638
T. albacares	C.....T.....C.....	641

Fig. 6. Alignment of *ITS1* sequences from the abnormal tuna (DDBJ/EMBL/GenBank accession number MW960117) Pacific bluefin (*Thunnus orientalis*, EU708983), bigeye (*T. obesus*, AB212025), and yellowfin (*T. albacares*, EU708982) tunas.

Abnormal tuna1	GCGCGCAACA-GACGCCCTCCGACAGCTGAGGGCCAGAGGGTAGGTCTCTTCCCCCCCCGGGTCCCGTCGCGGTCTCCCGAGGACTTCCCGCGCG	99
Abnormal tuna2G.....	99
T.orientalisG..T.....	99
T.obesusG..A.....G....CG.....-G.....	99
T.albacaresG..A.....G....CG.....G.....	100
Abnormal tuna1	CGGCGCGCGC--G-TGGTCGTTTCCCTCCCG-AAGGCCCGAGGCGCGCGC---CGGCGCTTTTCTTCCCGAGACGC---CCCGCGCAAAACACCTCTC	187
Abnormal tuna2C.....	187
T.orientalisC.....	187
T.obesusCG.-C.....-C.....CGTG.....C.....TCCC.....T.....	197
T.albacaresCG.GC.....TT.C.....CGTGG.....C.....T...TCCC.....	200
Abnormal tuna1	CTGGTCAGGACCTCGTCCCGACCGGGCGGGCCCT-CCCCACTTCTCCCCCCCCCTGGGCGACACGCCTCGCGACCCCGGCGAGCCTAAGCTCCCGGGGGCTG	286
Abnormal tuna2	286
T.orientalis	286
T.obesusT.....GT.....T.....G.....	297
T.albacaresT.....GT.....T.....AC.....T...G.....	299
Abnormal tuna1	ACGGAGCGG-CGGGTCCGACGTCGGGGCGCCCTCGGGGGGTTCCGAGCGCCCGGGGGTCCGGTGCACCGGGGCTGGGCGGACACTCCGA	370
Abnormal tuna2	370
T.orientalis	370
T.obesusG.....T.....C..T.....	381
T.albacaresG.....C..T.....	383

Fig. 7. Alignment of *ITS1* sequences from the abnormal tuna trial 1 (primers used from Chow and Kishino, 1995; DDBJ/EMBL/GenBank accession number MW960117), the abnormal tuna trial 2 (bigeye-tuna-specific primers used; OM851967), and Pacific bluefin (*Thunnus orientalis*, EU708983), bigeye (*T. obesus*, AB212025), and yellowfin (*T. albacares*, EU708982) tunas.



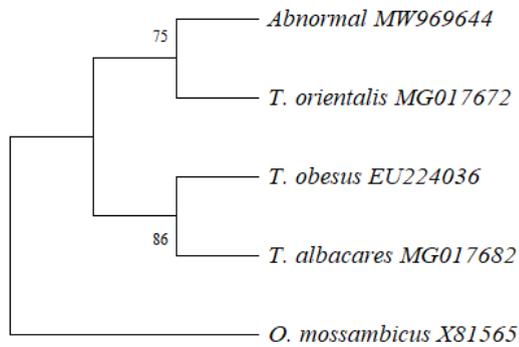


Fig. 8. Maximum Likelihood tree for the abnormal tuna and its three potential parent species, constructed using MEGA 11 based on *cyt b* DNA sequences aligned using Clustal Omega. Numbers on branches indicate bootstrap values. A cichlid, *Oreochromis mossambicus* Peters, 1852 (DDBJ/EMBL/GenBank accession 30 number X81565) was used as the outgroup.

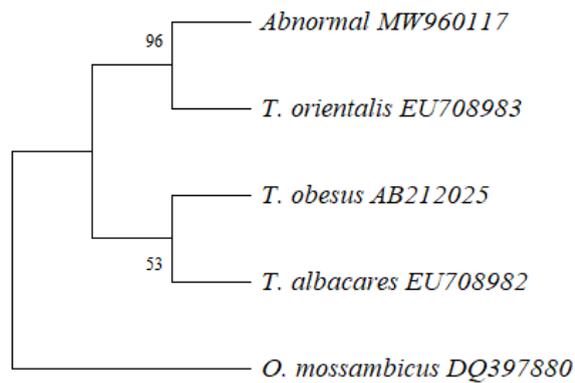


Fig. 9. Maximum Likelihood tree for the abnormal tuna and its three potential parent species, based on the DNA, and the tree was constructed using MEGA 11 based on *ITS1* sequences aligned using Clustal Omega. Numbers on branches indicate bootstrap values. A cichlid, *Oreochromis mossambicus* Peters, 1852 (DDBJ/EMBL/GenBank accession number DQ397880) was used as the outgroup.

Discussion

Traits and records

Tungkang in Pingtung County is the largest tuna landing port in Taiwan. Having landed and sold many bluefin, bigeye, and yellowfin tunas there for over 30 years, the members of the Tungkang Fishermen's Association are very familiar with the species of tuna found around Taiwan, none of which fully corresponds with this peculiar individual. Mr. Han-Chou Lin and many tuna-related people told media interviewers that it was the first time they had seen such a peculiar tuna with the characteristics of different species mixed in one individual. Bluefin tuna are characterised by a large body weighing about 100–300 kg, black skin with no yellow lines, black eyes, and short pectoral fins (Goujon and Majkowski, 2000). Bigeye tuna are characterised by a maximum weight of about 200 kg, large eyes, and long pectoral fins (Song et al., 2008). Yellowfin tuna are smaller, 40–80 kg, and characterised by yellow, sickle-like extensions of the second dorsal fin and anal fin, long, yellow pectoral fins, and ribbon-like stripes on the body surface (Appleyard et al., 2001). Features of the present abnormal tuna that matched bluefin tuna are the large body, black eyes, body depth (Fig. 2B), fat-rich meat, elastic abdominal flesh, and fat-lined abdomen (Fig. 3A). The meat also reportedly tasted like that of bluefin tuna. However, the body shape, pectoral fins, and catch season resembled those of bigeye or yellowfin tuna, while the anal fin, in particular, resembled that of bigeye tuna (Fig. 2C). Morphological criteria alone are clearly not enough to resolve the

identity of the abnormal tuna.

Mitochondrial and nuclear genes

The development of DNA-based mitochondrial and nuclear markers allows for better identification of morphologically similar fish species (Karaiskou et al., 2005) and hybrids (Gross et al., 1996). Nuclear genes allow the identification of interspecific hybrids (Porto-Foresti et al., 2013), and mitochondrial genes are particularly helpful for determining the direction of the cross because they represent contributions exclusively of the maternal lineage (Harrison, 1989). Although mitochondrial genes like *cyt b*, *COI* and 16S rRNA are often used as markers (genetic barcodes) to identify commercial fish (Rehbein, 2013), not all fish can be identified by this means. Humans have used hybridisation techniques to improve the biological characteristics of farmed fish, and hybridization makes identification difficult. Examples of indistinguishable species occur in often-hybridised fish such as striped bass, tilapia, catfish (Bartley et al., 2000), and various wild freshwater species (Schrey, 2011). In addition, because processed foods such as fillets, fish sticks, and surimi tend to lack useful morphological characteristics, analysis of mtDNA and nDNA is one of the few methods available to identify their ingredients (Rehbein, 2013).

Using the principle of maternal mitochondrial inheritance, the present *cyt b* and *COI* sequence data have confirmed that the mother of the present abnormal tuna was a bluefin tuna (Figs. 4, 5). The fact

that its maternal- and paternal-derived nuclear gene (*ITS1*) sequences were nearly identical to each other and to that of a reference bluefin tuna (Tables 1, 2), as furthermore confirmed using yellowfin-tuna-specific primers (Tables 1, 2), allows us to conclude that the father fish was also a bluefin tuna. The two-nucleotide difference for *ITS1* obtained for the abnormal fish in two sequencing trials (Fig. 7: abnormal tuna 1 and 2), may represent different sources (from maternal and paternal) of *ITS1*. Since both parents were bluefin tuna, we conclude that the abnormal tuna landed at Tungkang fishing port on 14 December 2020 was not a hybrid specimen, despite its morphological peculiarities. The question remains, though, of the cause of this specimen's unusual appearance. From the genetic analysis presented here, it is impossible to infer an answer.

As far as we know, the present abnormal tuna specimen is unique, but fisheries biologists and the tuna fishing industry itself should be alert to the possibility of more being found. The emergence of additional abnormal tuna in nature could signify a mutation favoured by environmental change, including human-mediated changes, something that may play a part in the future evolution of tuna species. If so, the advent of abnormal bluefin tuna may reflect the dual significance of environmental change and biological evolution (Grabenstein and Taylor, 2018).

Conclusion

In the beginning, based on photographs and ecological data on this abnormal tuna, we thought it was likely to be a hybrid and designed the study to investigate the option as the null hypothesis. However, the mitochondrial genes *cyt b* and *COI* showed that the maternal parent species of the abnormal tuna was a Pacific bluefin tuna (*Thunnus orientalis*), and the nuclear *ITS1* gene showed that the paternal parent was the same species. This conclusion rejects our null hypothesis but provides an answer to an issue that has sparked public debate in Taiwan.

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Author contributions: Ming-Chih Huang: Designed the study, data analysis, and manuscript writing.

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