Supplementary Table 1

Supplementary Table 1. All data of insemination tests

Date			Species							
Year	Month	Day	Female	Male	No. of eggs	Bouyancy (%)	No. of Bouyancy eggs	Fertilization (%)	Embryonic-body stage (%)	Hatching (%)
2013	10	24	ELT	ELT	3450	94.2	3250	83.8	76.8	74.0
			ELT	PBT	26000	100.0	26000	84.5	80.9	77.5
2013	10	31	ELT	ELT	10660	100.0	10660	80.6	77.9	77.9
			ELT	PBT	34660	100.0	34660	14.0	11.6	11.6
2013	11	15	ELT	ELT	13440	99.2	13333	90.0	78.3	65.0
			ELT	PBT	24880	96.5	24000	66.7	65.0	61.7
2015	10	4	ELT	ELT	13333	97.2	12963	68.1	56.9	52.7
			ELT	BT	47667	96.1	45829	62.1	0.0	0.0
			ELT	SJT	65333	96.9	63288	14.6	0.0	0.0
2015	10	8	ELT	ELT	38000	69.1	26258	62.8	57.4	53.3
			ELT	BT	35667	87.6	31230	39.6	0.0	0.0
			ELT	SJT	35000	78.8	27575	20.1	0.0	0.0
			ELT	LTT	60667	79.9	48453	53.0	44.3	36.7
2016	7	29	ELT	ELT	32450	94.5	30665	53.1	47.7	46.2
			ELT	ALT	110250	95.6	105350	28.3	26.1	24.8
2016	8	18	ELT	ELT	24560	100.0	24560	91.9	85.9	80.5
			ELT	ALT	135567	100.0	135567	27.1	21.1	20.5
2017	9	7	ELT	ELT	38383	97.9	37567	64.1	61.3	60.3
			ELT	ALT	29400	100.0	29400	23.3	22.2	21.3
2017	10	14	ELT	ELT	38383	97.9	37567	89.3	86.4	83.5
			ELT	ALT	60433	98.7	59617	28.4	25.8	23.1
2018	9	5	ELT	ELT	49500	100.0	49500	98.2	96.5	94.6
			ELT	ALT	123750	100.0	123750	31.8	29.6	28.9
2021	9	24	ELT	ELT	25317	67.7	17150	92.2	86.7	85.6
			ELT	LTT	46550	100.0	46550	20.8	16.0	12.3
2021	10	22	ELT	ELT	10200	100.0	10200	43.1	37.2	35.2
			ELT	LTT	53167	100.0	53167	41.8	32.5	18.6

Supplementary Table 2

Supplementary Table 2. Intraperitoneally transplantation of PBT testicular cells into HLT recipients

Trial	No. of transplanted	DAH at transplantation	Total length at transplantation (mm)	Incorporation rate	No. of incorporated PBT germ cells (range)	Age at sampling (m.o.)	No. of survive (%)	No. of matured male	No. of recipient producing PBT sperm (%)
1	2000	10–11	5.2–5.6	80.0 ($n = 5$)	3.5 ± 1.3 (1–8)	12	67 (3.4)	41	2 (4.9)
2	2700	9–10	5.3–5.8	40.0 ($n = 5$)	1.5 (1–2)	10	16 (0.6)	5	2 (40.0)
3	1600	8–9	5.3–5.8	73.3 ($n = 15$)	2.3 ± 0.4 (1–4)	8	43 (2.7)	22	5 (22.7)
Total	6300						126 (2.0)	68	9 (13.2)

Supplementary Table 3

Supplementary Table 3. Production efficiency of donor-derived PBT sperm in HLT recipients

Year of transplantation	Recipient age (months)	No. of PBT sperm produced recipient	Recipient ID	No. of analyzed sperm	No. of detected PBT sperm	Production efficiency
2016	12	2	#2016-55	5.0×10^{6}	2	$1/2.5\times10^{6}$
			#2016-82	5.0×10^{6}	1	$1/5.0\times10^{6}$
2018	10	2	#2018-48	5.0×10^{6}	2	$1/2.5\times10^{6}$
			#2018-60	5.0×10^{6}	1	$1/5.0\times10^{6}$
2019	8	5	#2019-5	5.0×10^{6}	1	$1/5.0\times10^{6}$
			#2019-9	5.0×10^{6}	1	$1/5.0\times10^{6}$
			#2019-10	5.0×10^{6}	1	$1/5.0\times10^{6}$
			#2019-21	5.0×10^{6}	1	$1/5.0\times10^{6}$
			#2019-22	5.0×10^{6}	1	$1/5.0\times10^{6}$

Supplementary Table 4

Supplementary Table 4. Estimation of cell division number of spermatogonium in PBT and HLT

Species	No. of spermatid in the largest-cross section in each of 10 different cysts		Estimated number of spermatid per cyst cell division
	Mean	Standard error	
Pacific bluefin tuna	132.9	5.5	2^{10}
Hybrid little tuna	133.6	3.9	2^{10}

Supplementary Table 5

Supplementary Table 5. Reference sequences with accession numbers

Gene	**Species**	**Nucleotide**
ddx4	Eastern little tuna *Euthynnus affnis*	OR917090
	Atlantic little tuna *Euthynnus alletteratus*	OR917089
	Pacific bluefin tuna *Thunnus orientalis*	EU253482
dead end	Eastern little tuna *Euthynnus affnis*	OR917092
	Atlantic little tuna *Euthynnus alletteratus*	OR917091
	Pacific bluefin tuna *Thunnus orientalis*	KF128758
co1 and D-loop	Eastern little tuna *Euthynnus affnis*	NC_025934
	Atlantic little tuna *Euthynnus alletteratus*	NC_004530
	Pacific bluefin tuna *Thunnus orientalis*	NC_008455

Supplementary Table 6

Supplementary Table 6. Primers

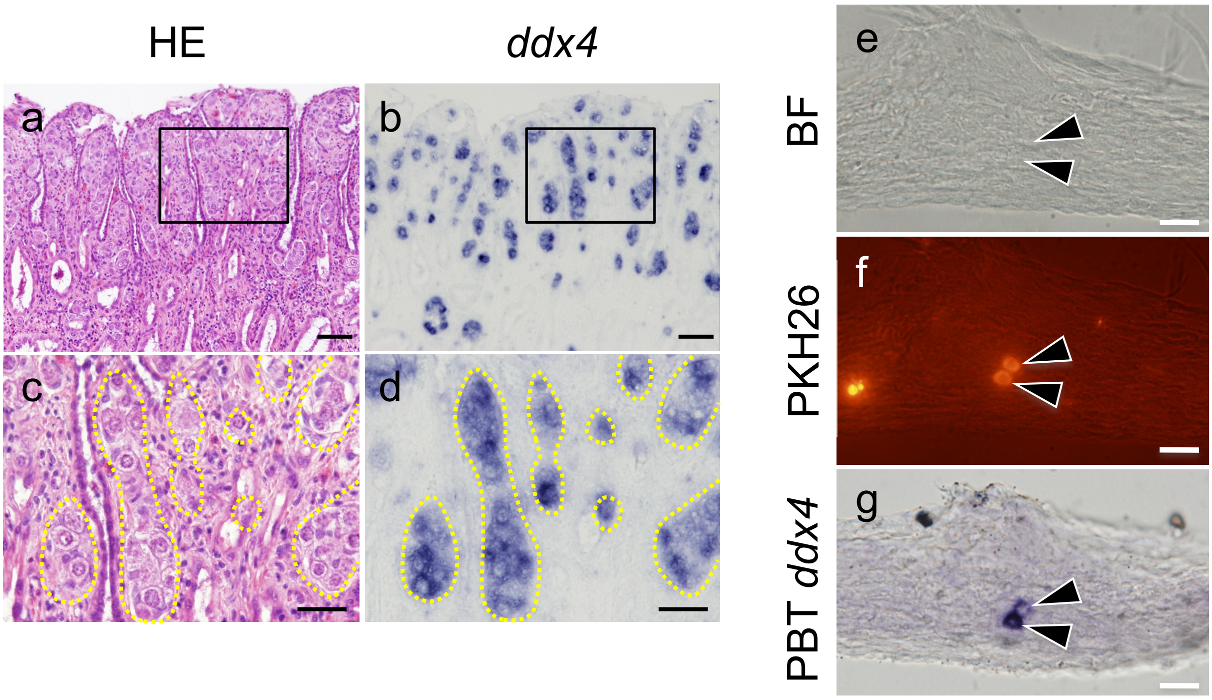
Purpose	Gene	Primer name	Sequence (5' to 3')
Hybrid check	*DEAD-box helicase 4 (ddx4)*	Suma_Yaito_checkvasa_F1	ATGACCCTGATGCTGATGGC
		Suma_Yaito_checkvasa_R1	GCAGACTCTTCTAACCATGAAGG
	cytochrome C oxidase subunit I (co1)	CO1_ELT_forHiDi_Fw	TTTTCTCCCTCCATCTAGCA
		CO1_ALT_forHiDi_Fw	TTTTCTCCCTCCATCTAGCG
		CO1_ELT-ALT_forHiDi_Rv	GGTCTGTCAGGAGCATTGTA
Detection of embryo derived from PBT sperm	*ddx4* (PBT-specific)	Tuna_vasa_Fn2	GTCCAGTCCTAGTAGCAACC
		Tuna_vasaRn_forSUM	GTAGCAGGTCCGCTGAACGC
	dead end (PBT-specific)	BFT_dnd_F1	CAGTGAAGCTGAGTCCGGAC
		BFT_dnd_R1	GATCCTGAGAATGCAGCAGA
	Ta-117 (PBT-specific)	Ta-117_F	TCACAGCATGGGACAAC
		Ta-117_R	ATAGTGAAATGATTAGAACAGTGC
	d-loop (PBT-specific)	BFT_Dloop_Fw2	CTTAACCATTCATATGTCAA
		BFT_Dloop_Rv1	GAAATGAGCTGGATGGTAGG
	ddx4 (common)	ddx4_common_F	GTAAATGTCCCGTCCTAGT
		ddx4_common_R	TCAGCATCAGGGTCATAAAA
	co1 (common)	CO1_common_F	CACACGCTGATTTTTCTC
		CO1_common_R	GGCCGTAACGATTACATT

Supplementary Table 7

Supplementary Table 7. PCR conditions

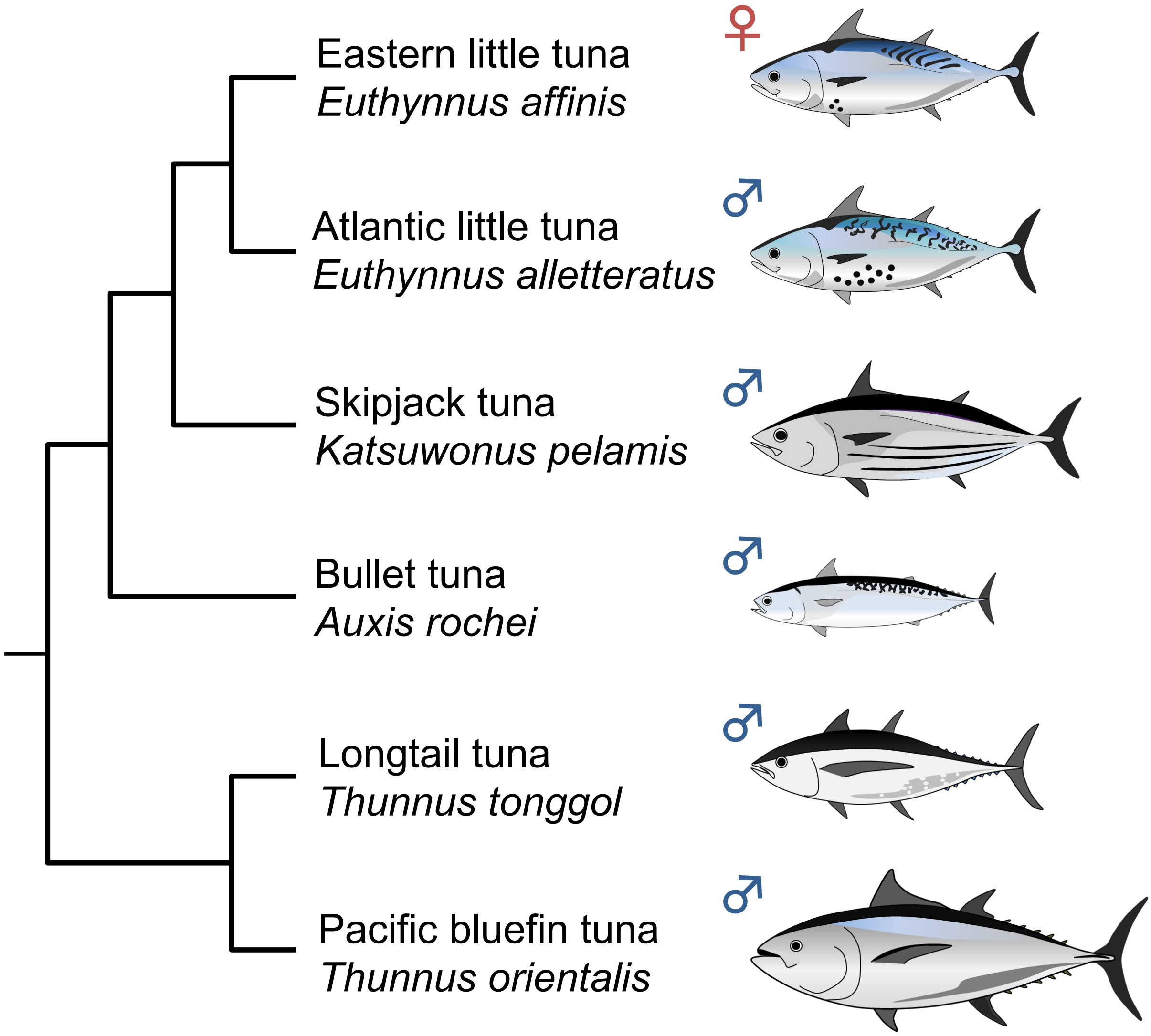
Purpose	Forward primer	Reverse primer	DNA polymerase	Initial denaturation (℃) (seconds)	Cycling: Denaturation (℃) (seconds)	Cycling: Annealing (℃) (seconds)	Cycling: Extention (℃) (seconds)	Cycling: Number of cycles	Final extension (℃) (seconds)
Hybrid check									
DEAD-box helicase 4 (ddx4)	Suma_Yaito_checkvasa_F1	Suma_Yaito_checkvasa_R1	TaKaRa Ex Taq	95 (180)	95 (30)	58 (30)	72 (60)	35	72 (180)
cytochrome C oxidase subunit I (co1)	CO1_ELT_forHiDi_Fw	CO1_ELT-ALT_forHiDi_Rv	HiDi DNA Polymerase	95 (180)	95 (15)	58 (15)	72 (30)	35	72 (180)
	CO1_ALT_forHiDi_Fw	CO1_ELT-ALT_forHiDi_Rv	HiDi DNA Polymerase	95 (180)	95 (15)	58 (15)	72 (30)	35	72 (180)
Detection of embryo derived from PBT sperm									
ddx4	Tuna_vasa_Fn2	Tuna_vasaRn_forSUM	Amplitaq gold DNA polymerase	95 (600)	95 (15)	60 (15)	72 (30)	35	72 (300)
dead end	BFT_dnd_F1	BFT_dnd_R1	Amplitaq gold DNA polymerase	95 (600)	95 (15)	58 (15)	72 (30)	35	72 (300)
Microsatelite locus	Ta-117_F	Ta-117_R	Amplitaq gold DNA polymerase	95 (600)	95 (15)	58 (15)	72 (30)	35	72 (300)
d-loop	BFT_Dloop_Fw2	BFT_Dloop_Rv1	Amplitaq gold DNA polymerase	95 (600)	95 (15)	58 (15)	72 (30)	35	72 (300)
internal control									
ddx4	ddx4_common_F	ddx4_common_R	TaKaRa Ex Taq	95 (180)	95 (30)	58 (30)	72 (60)	35	72 (180)
co1	CO1_common_F	CO1_common_R	TaKaRa Ex Taq	95 (180)	95 (30)	58 (30)	72 (60)	35	72 (180)

Supplementary Fig. 1



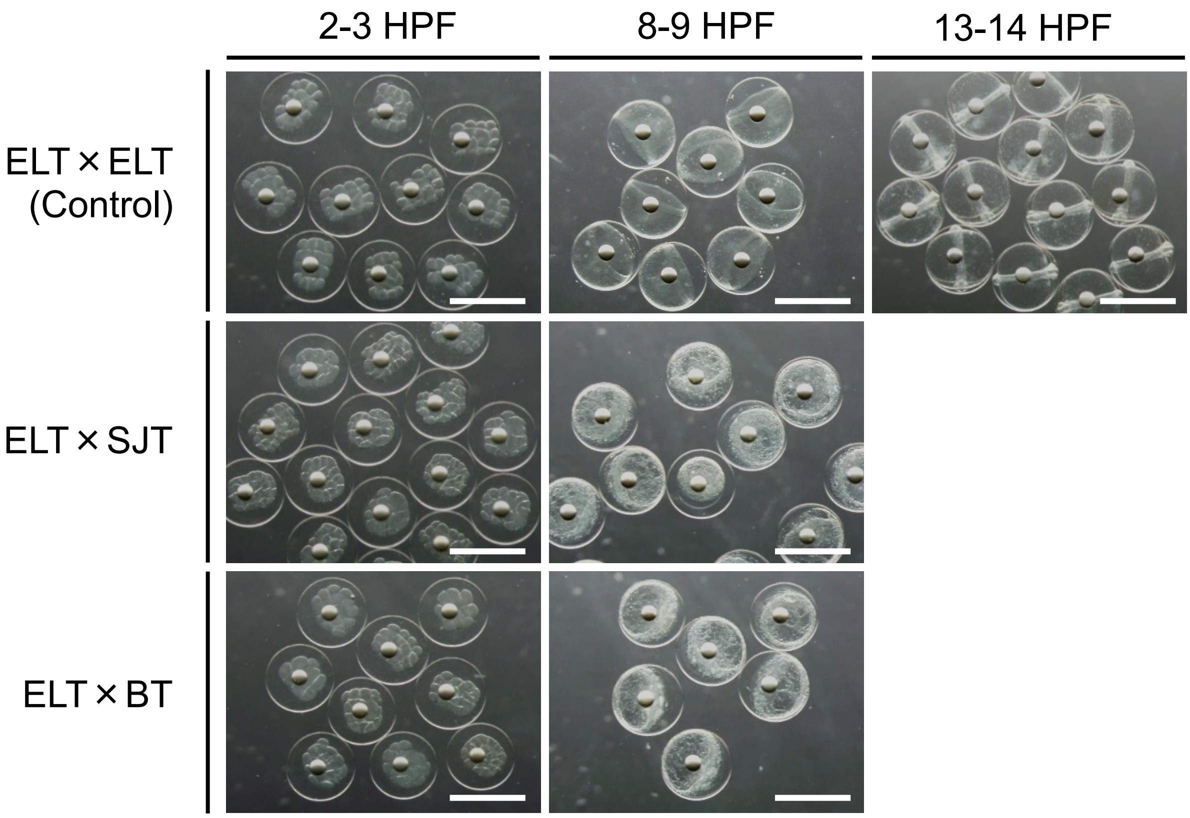
Supplementary Fig. 1 Transplantation of prepubertal 1-year-old Pacific bluefin tuna (PBT) germ cells into eastern little tuna (ELT) recipients. (a–d) Sequential sections of donor testis obtained from a 1-year-old PBT (total length, 61 cm; body weight, 3.2 kg) subjected to hematoxylin and eosin staining (a,c) and *in situ* hybridization (ISH) with *ddx4* probe (b,d). High-magnification views of the boxed areas in (a) and (b) are shown in (c) and (d), respectively. Yellow broken lines indicate colonized *ddx4*-positive type A spermatogonia. Bars = 40 µm (a,b) and 20 µm (c,d). **(e–f)** Incorporation of 1-year-old PBT germ cells stained with red fluorescence dye (PKH 26) into the gonad of an ELT recipient at seven days after transplantation. Columns, top to bottom: bright-field view (e), fluorescent view (f), and ISH with PBT-specific *ddx4* 3′ untranslated regions (g) of the excised recipient gonad. Black arrowheads: incorporated donor PBT germ cells. In this experiment, 2 of 22 ELT recipients (9.1%) incorporated donor-derived PBT germ cells. Representative images are shown from three biologically independent PBT testis (a-d) and two ELT recipients incorporated PBT germ cells (e-g). Bars = 20 µm.

Supplementary Fig. 2



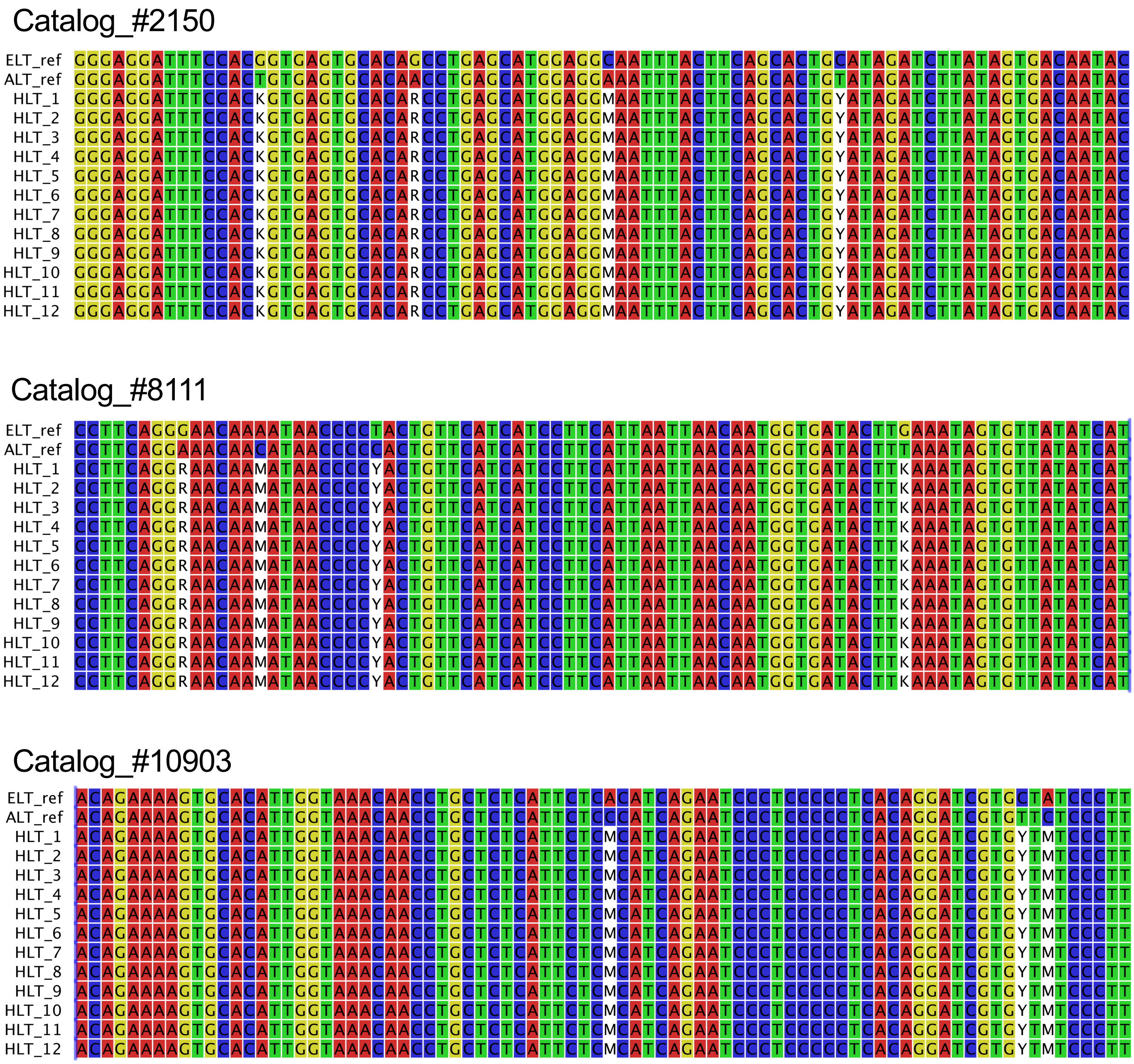
Supplementary Fig. 2 Hypothesized phylogenetic relationships among Eastern little tuna and their closely related species used for interspecific hybridization. Redrawn from (Miya et al. 2013; Díaz-Arce et al. 2016).

Supplementary Fig. 3



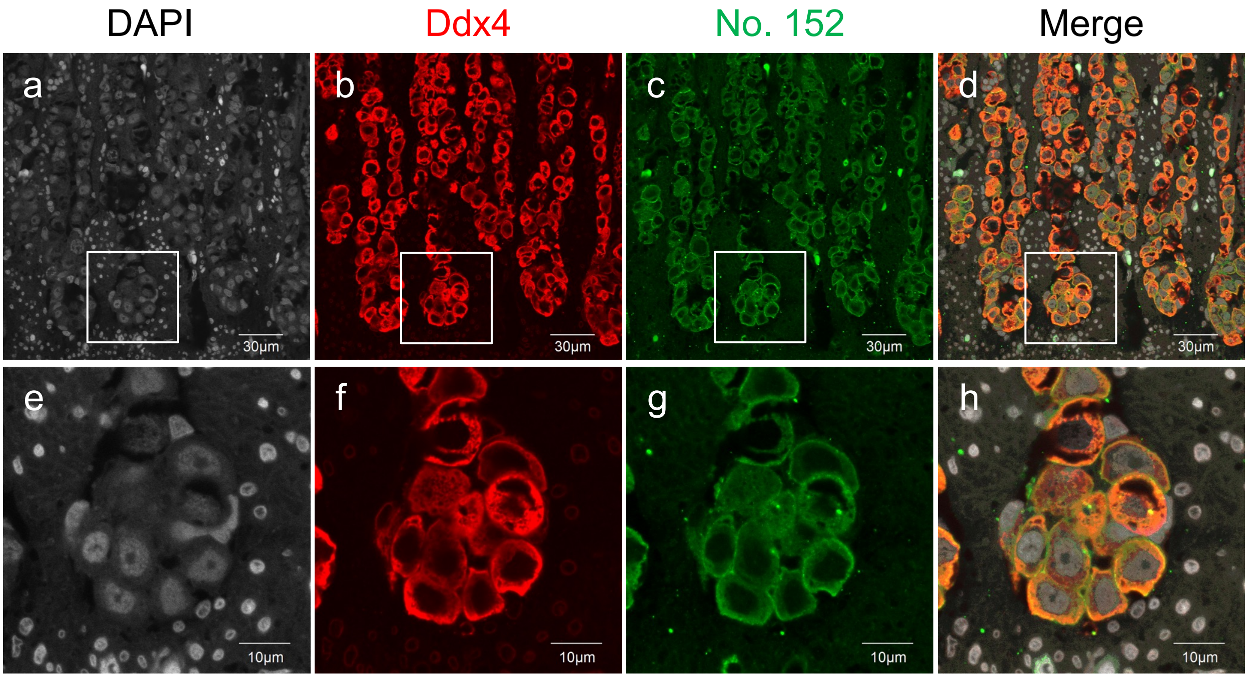
Supplementary Fig. 3 Micrograph of control ELT, ELT × SJT, and ELT × BT hybrids at 16–32-cell (2–3 hour-post fertilization, HPF; left column), epiboly (8–9 HPF; middle column), and embryonic body stage (13–14 HPF; right column). The embryo development of ELT × SJT and ELT × BT hybrids was arrested at the epiboly stage. Bars = 200 μm.

Supplementary Fig. 4



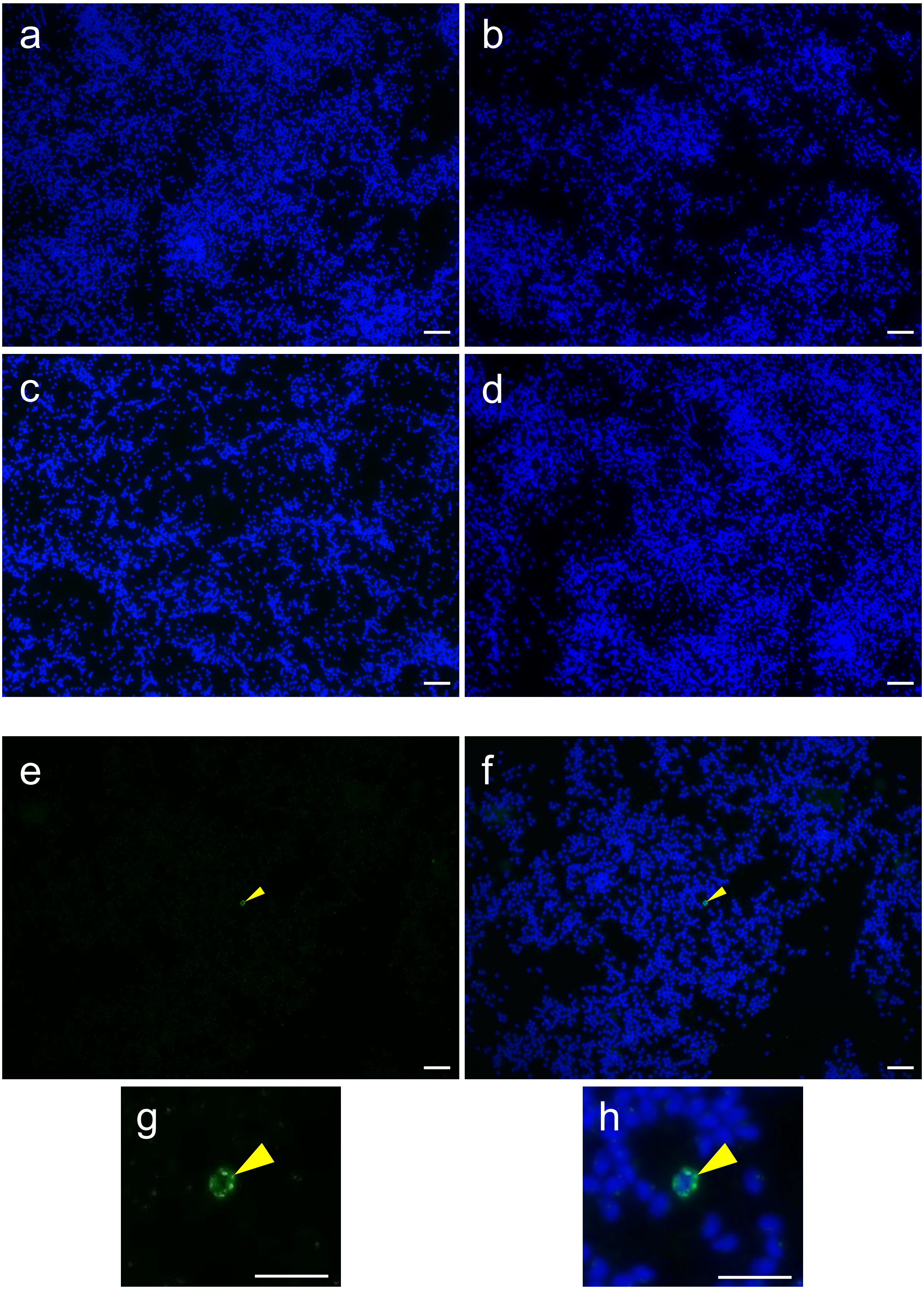
Supplementary Fig. 4 Typical examples of single nucleotide polymorphisms (SNPs) carrying both parental species alleles found in the HLT genome. Top to bottom: Consensus sequences derived from pooled DNA of eastern little tuna (*n* = 7), Atlantic little tuna (*n* = 5), and each DNA of 12 hybrid little tuna individuals on catalog sequences No. 2,150, No. 8,111, and No. 10,903. All catalog sequences and SNP information are shown in Supplementary Data 1–3.

Supplementary Fig. 5



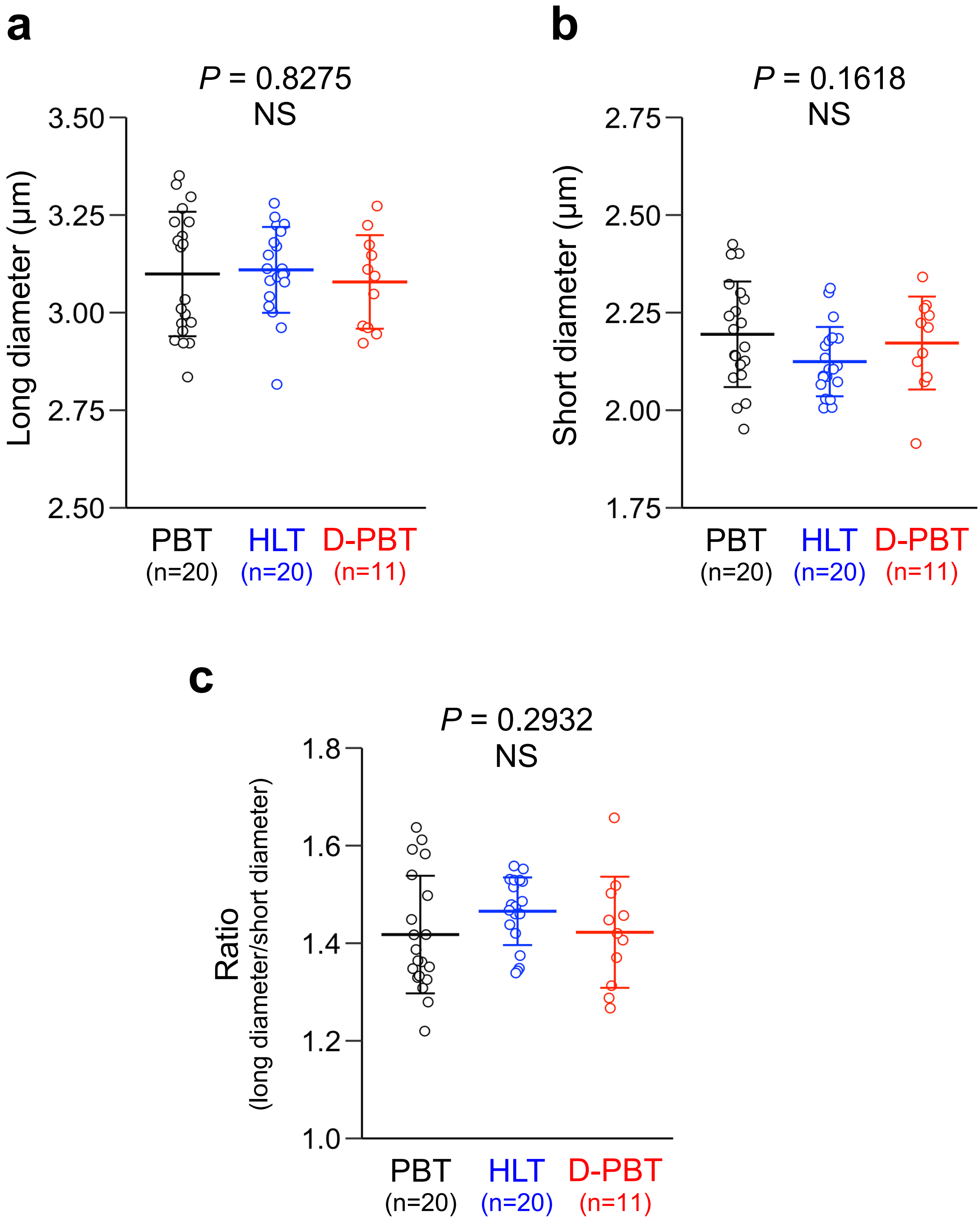
Supplementary Fig. 5 Double immunohistochemistry of donor Pacific bluefin tuna (PBT) testis using anti-Ddx4 antibody and No. 152 antibody. (a–h) Columns, left to right: DAPI (a and e), anti-Ddx4 antibody (germ cell marker [b and f]), No. 152 antibody (type A spermatogonia marker, Ichida et al. 2019 [c and g]), and a merged view (d and h). High-magnification views of the boxed area in (a)–(d) are shown in (e)–(h) respectively. A large number of Ddx4 and No. 152 double-positive type A spermatogonia containing germline stem cells were present in the donor PBT testis used in this study. Representative images from three biologically independent samples. Bars = 30 μm (a–d) and 10 μm (e–h).

Supplementary Fig. 6



Supplementary Fig. 6 Specificity of immunocytochemistry (ICC) using Pacific bluefin tuna (PBT) sperm–specific antibody No. 149. (a–d) Low-magnification views of No. 149 staining (green) merged with DAPI (blue) for sperm smears derived from four different individuals (a–d) of nontransplanted hybrid little tuna (HLT). **(e–h)** Low-magnification views of No. 149 staining against a sperm smear of nontransplanted HLT sperm mixed with PBT sperm at a ratio of 1,000,000:1. Left panels: No. 149 staining (green). Right panels: merged view with DAPI (blue). High-magnification views of (e)–(f) are shown in (g)–(h) respectively. Yellow arrowheads indicate PBT sperm detected by the PBT sperm–specific antibody. Bars = 20 μm (a and upper b) and 10 μm (lower b).

Supplementary Fig. 7



Supplementary Fig. 7 Morphology analysis of donor-derived Pacific bluefin tuna sperm (D-PBT) in recipient milt detected by immunocytochemistry. a–c Long diameter (a), short diameter (b), and the ratio of the long to the short diameter (c) of sperm heads on sperm smears. Control PBT sperm, $n = 20$; control HLT sperm, $n = 20$; donor-derived PBT sperm, $n = 11$. Data were analysed by one-way analysis of variance. All data are shown as means ± standard deviation. NS, $P > 0.05$.

Supplementary Fig. 8

a

```
                               20                                    40                                    60
No.8_ddx4  GTCCAGTCCT AGTAGCAACC TCTGTAGCTG CCCGCGGTCT GGATATTCCA GATGTACAGC  60
 PBT_ddx4  GTCCAGTCCT AGTAGCAACC TCTGTAGCTG CCCGCGGTCT GGATATTCCA GATGTACAGC  60
 ELT_ddx4  GTCCCGTCCT AGTGGCAACA TCTGTAGCTG CCCGCGGTCT GGATATTCCA GATGTGCAGC  60
 ALT_ddx4  GTCCCGTCCT AGTGGCAACA TCTGTAGCTG CCCGCGGTCT GGATATTCCA GATGTGCAGC  60
                               80                                   100                                   120
No.8_ddx4  ATGTGGTTAA CTTTGACCTC CCCAACAACA TTGATGAATA TGTCCACCGT ATTGGGAGGA 120
 PBT_ddx4  ATGTGGTTAA CTTTGACCTC CCCAACAACA TTGATGAATA TGTCCACCGT ATTGGGAGGA 120
 ELT_ddx4  ATGTGGTGAA CTTTGACCTC CCCAACAACA TTGATGAATA TGTCCACCGT ATTGGGAGAA 120
 ALT_ddx4  ATGTGGTGAA CTTTGACCTC CCCAACAACA TTGATGAATA TGTCCACCGT ATTGGGAGAA 120
                              140                                   160                                   180
No.8_ddx4  CTGGCCGCTG CGGTAACACA GGGAGGGCAG TGTCTTTCTA TGACCCTGAT GCTGATGGCC 180
 PBT_ddx4  CTGGCCGCTG CGGTAACACA GGGAGGGCAG TGTCTTTCTA TGACCCTGAT GCTGATGGCC 180
 ELT_ddx4  CCGGCCGCTG CGGTAACACT GGGAGGGCAG TGTCTTTTTA TGACCCTGAT GCTGATGGCC 180
 ALT_ddx4  CTGGCCGCTG CGGTAACACT GGGAGGGCGG TGTCTTTTTA TGACCCTGAT GCTGATGGCC 180
                              200                                   220                                   240
No.8_ddx4  AACTGGCTCG CTCCTTGGTC ACAGTCCTGT CCAAGGTAAG AGCTGTCTGC TCCGGAAAGT 240
 PBT_ddx4  AACTGGCTCG CTCCTTGGTC ACAGTCCTGT CCAAGGTAAG AGCTGTCTGC TCCGGAAAGT 240
 ELT_ddx4  AACTGGCTCG CTCCTTGGTC ACAGTCCTGT CCAAGGTAAG ---------- --------GT 222
 ALT_ddx4  AACTGGCTCG CTCCTTGGTC ACAGTCCTGT CCAAGGTGAG ---------- --------GT 222
                              260                                   280                                   300
No.8_ddx4  CATCTGGAGA GCACCAACCT TATATTACCA TTCACATCAA GCAGTAATTG GAATATTTAT 300
 PBT_ddx4  CATCTGGAGA GCACCAACCT TATATTACCA TTCACATCAA GCAGTAATTG GAATATTTAT 300
 ELT_ddx4  CATCTGGATA CT--TAACCT TACATCATCA TTCACATTAA GCAGTAATTG GAATATTCAT 280
 ALT_ddx4  CATCTGGATA CT--TAACCT TACATCATCA TTCACATCAA GCAGTAATTG GAATATA--- 277
                              320                                   340                                   360
No.8_ddx4  ACATTCAGTG GGGTCCAAAA GTCTAAGACC ACTTTCCCAT TTACTTGAAT GAGAAAGTGG 360
 PBT_ddx4  ACATTCAGTG GGGTCCAAAA GTCTAAGACC ACTTTCCCAT TTACTTGAAT GAGAAAGTGG 360
 ELT_ddx4  ACATACTGTG GGCTCCAAAA GTCTGAGACC ACTT------ ---------- ---------- 314
 ALT_ddx4  ---------- ---------- ---------- ---------- ---------- ---------- 277
                              380                                   400                                   420
No.8_ddx4  TCTCAGACTT TTGGAACCAC TTGGAAGGAC TTCAAGCACA GTCAGTGTCA GCTTACATTG 420
 PBT_ddx4  TCTCAGACTT TTGGAACCAC TTGGAAGGAC TTCAAGCACA GTCAGTGTCA GCTTACATTG 420
 ELT_ddx4  ---------- ---------- ---GGAGGAC TTGAAGCACA GTCAGTGTCC GCTTTCATTG 351
 ALT_ddx4  ---------- ---------- ---------- ---------- ---------- ----TTATGT 283
                              440                                   460                                   480
No.8_ddx4  TTTCCTCATG TTGATTGGGA TTTTCAGGCC CAGCAGGAAG TGCCTTCATG GTTAGAAGAG 480
 PBT_ddx4  TTTCCTCATG TTGATTGGGA TTTTCAGGCC CAGCAGGAAG TGCCTTCATG GTTAGAAGAG 480
 ELT_ddx4  TTTCCTTATG TTGATTGGGA TTTACAGGCC CAGCAGGAAG TGCCTTCATG GTTAGAAGAG 411
 ALT_ddx4  TTCCTTTATG TTGATTGGGA TTTACAGGCC CAGCAGGAAG TGCCTTCATG GTTAGAAGAG 343
                              500
No.8_ddx4  TCTGCGTTCA GCGGACCTGC TAC 503
 PBT_ddx4  TCTGCGTTCA GCGGACCTGC TAC 503
 ELT_ddx4  TCTGCATTCA GTGGACCTGC TGT 434
 ALT_ddx4  TCTGCATTCA GTGGACCTGC TGT 366
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b

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                              20                                    40                                    60
No.8_dnd  CAGTGAAGCT GAGTCCGGAC GACCCACAGC AGCCCTCTAA AAGCCTCCTG CCATCACCCC  60
 PBT_dnd  CAGTGAAGCT GAGTCCGGAC GACCCACAGC AGCCCTCTAA AAGCCTCCTG CCATCACCCC  60
 ELT_dnd  CAACGAAGCC GAGCCCAGAT GACCCACAGC AGCCCTCTAA AAGCCCCCTG CCATCACCCC  60
 ALT_dnd  CAACGAAGCC GAGTCCAGAC GACCCACAGC AGCCCTCTAA AAGCCCCCTG CCATCACCCC  60
                              80                                   100                                   120
No.8_dnd  TGAAGCCGCC GCGCCACATC CTGAACTCCC CGCAGCCCTC AGTCCTGCCT CCTC------ 114
 PBT_dnd  TGAAGCCGCC GCGCCACATC CTGAACTCCC CGCAGCCCTC AGTCCTGCCT CCTC------ 114
 ELT_dnd  TGAAGCCGCC GCACCCCATC CTCAACTCTC CGCAGCCCTC AGTCCCGCCT CCTCCTC--- 117
 ALT_dnd  TGAAGCCACC GCACCACATC CTCAACTCTC CGCAGCCCTC AGTCCCGCCT CCTCCTCCTC 120
                             140                                   160                                   180
No.8_dnd  ---GCCTGAT CCGCCCTCCA TCCATGCCCC CAGGTTTCTG CAGAGCAGTG GGAGGACCCA 171
 PBT_dnd  ---GCCTGAT CCGCCCTCCA TCCATGCCCC CAGGTTTCTG CAGAGCAGTG GGAGGACCCA 171
 ELT_dnd  ---GCCTGGT CCGCCCTCTG TCCGTCCCCC CAGGTTTCTG CAGAGCAGTG GGGGGACCCT 174
 ALT_dnd  CTCGCCTGGT CCGCCCTCTG TCCGTCCCCC CAGGTTTCTG CAGAGCAGTG GGGGGACCCA 180
                             200                                   220                                   240
No.8_dnd  CGGCTCCCCA GCACCCTCAC CCTCACCCTC CCTGCTCCTC CACCTACCAG GAGCATCTGT 231
 PBT_dnd  CGGCTCCCCA GCACCCTCAC CCTCACCCTC CCTGCTCCTC CACCTACCAG GAGCATCTGT 231
 ELT_dnd  CAGCCCCCCA GCACCGTCAC CCTC------ CCTGCTCCTC CACCTACCAG GGGCATCTGC 228
 ALT_dnd  CAGCCCCCCA GCACCGTCAC CCTC------ CCTGCTCCTC CACCTACCAG GGGCACCTGC 234

No.8_dnd  CTGCTGCATT CTCAGGATC 250
 PBT_dnd  CTGCTGCATT CTCAGGATC 250
 ELT_dnd  CCTCAGCATT CTCGGGTTC 247
 ALT_dnd  CCTCGGCATT CTCGGGTTC 253
```

Supplementary Fig. 8 Sequencing analysis of polymerase chain reaction (PCR) products derived from Pacific bluefin tuna (PBT)–positive sample No. 8. (a,b) Consensus sequence of PCR products amplified from sample No. 8: PBT, eastern little tuna, and Atlantic little tuna using primers targeting genomic region of *ddx4* (a) and *dnd* (b). The pink box indicates the different residues.