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Phylogenetic Relationships Between Tuna Species of the Genus *Thunnus* (Scombridae: Teleostei): Inconsistent Implications from Morphology, Nuclear and Mitochondrial Genomes

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Abstract. In order to infer phylogenetic relationships between tuna species of the genus *Thunnus,* partial sequences of the mitochondrial cytochrome *b* and ATPase genes were determined in all eight species. Supplemental restriction analysis on the nuclear rRNA gene was also carried out. Pacific northern bluefin tuna (*Thunnus thynnus orientalis*) was found to have mtDNA distinct from that of the Atlantic subspecies (*T. t. thynnus*) but very similar to that from the species albacore (*T. alaluga*). In contrast, no differentiation in nuclear genome was observed between the Atlantic and Pacific northern bluefin tunas. The Atlantic northern bluefin and southern bluefin tunas possessed mtDNA sequences very similar to species of yellowfin tuna group and not so similar to albacore and bigeye tunas which were morphologically assigned to the bluefin tuna group. The molecular data indicate that (1) mtDNA from albacore has been incorporated into the Pacific population of northern bluefin tuna and has extensively displaced the original mtDNA, and (2) albacore is the earliest offshoot, followed by bigeye tuna in this genus, which is inconsistent with the phylogenetic relationships between these tuna species inferred from morphology.

Key words: Tuna — Mitochondrial DNA transfer — Phylogenetic analysis

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Introduction

The systematics of the large tuna species of the genus *Thunnus* are now well understood (Iwai et al. 1965; Nakamura 1965; Gibbs and Collette 1967; Collette 1978; Collette and Nauen 1983). It has been agreed that the eight nominal species are separated into the yellowfin and bluefin tuna groups on the basis of anatomical characteristics. Collette (1978) considered that absence of the central heat exchanger in the species of the bluefin tuna group and presence in those of the yellowfin tuna group are significant keys for separating these groups. In the bluefin tuna group the livers consist of three subequal lobes covered with ventral striations caused by blood vessels and the red muscle is internalized. The right lobe of liver is the longest with no ventral striations and more orientation of red muscle to the lateral surface is observed in the yellowfin tuna group (Gibbs and Collette 1967; Collette 1978). Furthermore, several other anatomical characteristics of the cutaneous artery position and the axial skeleton are also noted to be significant in grouping these tuna species (Nakamura 1965; Gibbs and Collette 1967; Collette 1978). Tropical species, blackfin (*T. atlanticus*), longtail (*T. tonggol*), and yellowfin (*T. albacares*) tunas, are the members of the yellowfin group. The bluefin group contains the albacore (*T. alalunga*), the bigeye (*T. obesus*), and the northern bluefin (*T. thynnus*) and southern bluefin (*T. maccoyii*) tunas, which inhabit cooler waters. The northern bluefin tuna occurs infrequently in the Southern Hemisphere and the Indian Ocean, where the southern bluefin tuna dominates

Table 1. Tuna species used in this study

Species	Common name	Catch locality and date	Abbreviation
Thunnus alalunga	Albacore	North Pacific, 1991	ALB
T. albacares	Yellowfin tuna	North Pacific, 1992	YFT
T. atlanticus	Blackfin tuna	Western Atlantic, 1992, 1994	BFT
T. maccoyii	Southern bluefin tuna	South Indian Ocean, 1991	SBT
T. obesus	Bigeye tuna	North Pacific, 1991	BET
T. thynnus orientalis	Northern bluefin tuna	North Pacific, 1991, 1993	PNB
T.t. thynnus	Northern bluefin tuna	North Atlantic, 1992 Mediterranean, 1994	ANB
T. tonggol	Longtail tuna	South China Sea, 1992	LTT
Katsuwonus pelamis	Skipjack tuna	Eastern Indian Ocean, 1990	SKJ

(Collette and Nauen 1983). The apparent allopatric distributions but lack of morphological distinction between the Atlantic and Pacific northern bluefin tunas have made taxonomists to consider them two subspecies (*T. thynnus thynnus* and *T. t. orientalis*, respectively) (Jones and Silas 1960; Iwai and Nakamura 1964; Iwai et al. 1965; Gibbs and Collette 1967). Collette (1978) suggested that the species of the bluefin group have evolved from the tropical stem and adapted into cooler waters. This phylogenetic implication, however, has been questioned by several molecular analyses. For identifying species of the genus *Thunnus,* Chow and Inoue (1993) performed restriction-fragment-length polymorphism (RFLP) analysis on mitochondrial cytochrome *b,* 12S rRNA, and ATPase genes amplified by polymerase chain reaction (PCR), and found the Pacific northern bluefin tuna to share a much larger number of restriction fragments with albacore than with its Atlantic counterpart. Furthermore, Atlantic northern bluefin and southern bluefin tunas were observed to have restriction profiles more similar to those of the tropical species of the yellowfin tuna group than to those of the Pacific northern bluefin tuna, albacore, and bigeye tuna, which are assigned to be members of the bluefin group. Sharp and Pirages (1978) and Elliott and Ward (1995), using allozyme analysis, observed the albacore to be the most divergent species in the genus. Interestingly, Elliott and Ward (1995) reported that the bluefin tunas were much closer to the yellowfin tuna than to albacore and bigeye tuna. Finnerty and Block (1995) also reported that the mitochondrial cytochrome *b* data are not consistent with the taxonomic subdivision in the genus *Thunnus,* and suggested that conclusions about phylogenetic relationships between species may be drawn by obtaining mtDNA data from the two tropical species. At present, partial nucleotide sequencing of mitochondrial cytochrome *b* gene has been carried out in five species of the genus *Thunnus* (Bartlett and Davidson 1991; Block et al. 1993; Finnerty and Block 1995), but two tropical species (*T. atlanticus* and *T. tonggol*) of the yellowfin tuna group and Pacific northern bluefin tuna were not examined.

MtDNA has a fast rate of sequence evolution (Brown et al. 1982), and hence the sequence analysis is believed to be a powerful tool for inferring phylogenetic relationships between closely related species. Nevertheless, reliance on a single molecular, such as mtDNA, may result in construction of a false phylogeny and poor estimates of evolutionary rates from the molecular data, especially when gene introgression across a species boundary has occurred. Natural hybridization is believed to be more common in fish than in any other group of vertebrates (Campton 1987), and numbers of gene introgressions have been reported in fish (Smith 1992).

We have carried out partial nucleotide sequencing not only of the cytochrome *b* gene of the Pacific northern bluefin tuna and the two tropical tuna species, but also of the mitochondrial ATPase genes of all *Thunnus* species. Furthermore, we have carried out RFLP analysis on nuclear rRNA gene amplified by PCR. In this paper, we present molecular evidence on mtDNA transfer from albacore to the Pacific population of northern bluefin tuna and discuss evolutionary history among these tuna species based on the molecular data.

Materials and Methods

Tuna Samples: Tuna species and source of collection are listed in Table 1. Species names are abbreviated as: ALB (albacore; *Thunnus alalunga*), ANB (Atlantic northern bluefin tuna; *T. thynnus thynnus*), BET (bigeye tuna; *T. obesus*), BFT (blackfin tuna; *T. atlanticus*), LTT (longtail tuna; *T. tonggol*), PNB (Pacific northern bluefin tuna; *T. thynnus orientalis*), SBT (southern bluefin tuna; *T. maccoyii*), SKJ (skipjack tuna; *Katsuwonus pelamis*), and YFT (yellowfin tuna; *T. albacares*). Total DNA was extracted from muscle of fresh or frozen fish or ethanol-preserved muscle tissue. Procedures for total DNA extraction are described elsewhere (Chow et al. 1993; Chow and Inoue 1993).

DNA Amplification, restriction analysis, and nucleotide sequencing. PCR amplifications were performed in 25-μl volumes of Tris buffer (67 mM, pH 8.8) containing 2 mM $MgCl_2$, 1 mM of each dNTP, 1 μM of each primer, 50–500 ng template DNA, and 1 unit of *Taq* polymerase (Cetus). The primers used for mitochondrial cytochrome *b* gene (cytb) were abbreviated forms of conserved primers reported by Kocher et al. (1989), and the sequences are 5′-GCTTCCATCCAACATCTCAGCATGATG-3′ (L14838) and 5′-GCAGCCCCTCAGAATGATATTTGTCCTC-3′ (H15150). Primers for amplifying mitochondrial ATPase gene (ATP) were designed by Chow and Inoue (1993), and the sequences are 5′-CTTCGACCAATTTATGAGCCC-3′

(L8562) and 5′-GCCATATCGTAGCCCTTTTTG-3′ (H9432). Reaction mixtures to amplify these mitochondrial genes were preheated at 95°C for 2 min followed by 30 cycles of amplification (at 95°C for 1 min, 50°C for 30 s, and 72°C for 1.5 min). Primers to amplify internal transcribed spacer 1 (ITS1) in the nuclear rRNA gene family were designed from sequences of 18S and 5.8S rRNA genes reported by Clark et al. (1984), Medlin et al. (1988), and Tautz et al. (1988), and the sequences are 5′-GTCGTAACAAGGTTTCCGTA-3′ (ITS1F) and 5′-GTGCCGAGTGATCCACCGCT-3′ (ITS1R). ITS1 fragments were amplified using the same cycles for the mitochondrial genes but annealing temperature was set at 62°C.

Restriction analysis was performed on an ATP fragment of *T. thynnus thynnus* (ANB) and *T. t. orientalis* (PNB) and on an ITS1 fragment of all *Thunnus* species. Amplified fragments were digested using four base cutters, electrophoresed on a 2.5% agarose gel (BIOGEL, BIO101), and stained with EtBr for photographing. Nine out of 20 endonucleases used are reported to detect diagnostic restriction profiles for separating *T. t. thynnus* (ANB) and *T. t. orientalis* (PNB) (Chow and Inoue 1993), of which *Sau*961 was used to digest ATP fragments. Endonucleases used for RFLP analysis on ITS1 fragment were *Bsa*JI, *Dde*I, *Fnu*4HI, *Hae*III, *Mse*I, *Mbo*I, *Sau*96I, and *Taq*I.

Cytb and ATP fragments of *Thunnus* species and *K. pelamis* (SKJ) were purified using Gene Clean II (BIO 101). Purified DNA templates (150 ng for cytb and 400 ng for ATP) were subjected to direct nucleotide sequencing of double-stranded PCR products using a Taq dye-terminator cycle sequencing kit (ABI) for 26 cycles (at 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min). Primers used for sequencing cytb and ATP fragments were L14838 and L8562, respectively. Whenever possible the same individual was used for sequencing both genes.

Phylogenetic Analysis. Cytochrome *b* gene sequence data of *T. alalunga* (ALB), *T. t. thynnus* (ANB), *T. obesus* (BET), and *T. albacares* (YFT) were cited from Bartlett and Davidson (1991), and they were of Atlantic origin. Cytochrome *b* gene of two individuals of *T. alalunga* (ALB) from the Pacific were also sequenced. Sequences were aligned and the percentages of nucleotide substitutions between sequences were calculated using the Clustal V program with unweighted transitions (Higgins et al. 1992). Phylogenetic trees were constructed using the neighbor-joining (NJ) method (Saitou and Nei 1987) based on the percent of nucleotide substitutions, and the reliability of each interior branch was tested by 1,000 bootstrap replications using the Clustal V program. We also carried out a maximum likelihood procedure (ML), applying DNAML in PHYLIP version 3.5 (Felsenstein 1994) to a subset of the sequences (one sequence from each species). This procedure does not assume the constant rate of substitution and takes account of two types of substitution, transition and transversion (Hasegawa et al. 1985). The default value 2 was adopted for the transition/transversion ratio, and the results did not vary much for different values of the ratio. We did not take account of different rates among sites, because the sequences we studied were closely related. When the sequences are distant among others so that multiple substitutions along lineages are not negligible, analysis taking account of heterogeneity becomes crucial. To do the exhaustive search and comparison of topologies, we also applied NucML in MOLPHY (Adachi and Hasegawa 1994). The log likelihood ratio was approximated by a normal distribution according to Kishino and Hasegawa (1989).

Results

RFLP Between Atlantic and Pacific Northern Bluefin Tunas

Restriction patterns of ATP fragments of *T. t. thynnus* (ANB) and *T. t. orientalis* (PNB) digested by *Sau*96I are shown in Fig. 1, where the distinct restriction profiles between the two morphs are evident. Thirty-five individuals of *T. t. thynnus* (ANB) and 50 of *T. t. orientalis* (PNB) were analyzed, in which one individual of *T. t. orientalis* (PNB) represented identical restriction pattern with that of *T. t. thynnus* (ANB). The other 49 individuals of *T. t. orientalis* (PNB) showed restriction profiles identical to *T. alalunga* (ALB). The Atlantic-like sort of individual found in the *T. t. orientalis* (PNB) sample was designated PNB1 and treated separately from the other *T. t. orientalis* (PNB) individuals (albacore-like type) in the subsequent nucleotide sequence and phylogenetic analyses.

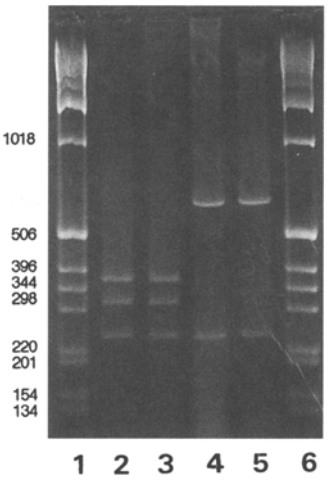

Fig. 1. EtBr-stained mitochondrial ATPase fragments of *T. thynnus orientalis* (PNB) (*lanes 2 and 3*) and *T. t. thynnus* (ANB) (*lanes 4 and 5*) digested by *Sau*96I. *Lanes 1 and 6* are 1-kb DNA ladder (Gibco, BRL) and the sizes in base pairs are shown at the *left.*

Nucleotide Sequences and Phylogenetic Analysis of mtDNA

The alignments of nucleotide sequences of the cytb and ATP are presented in Figs. 2 and 3, respectively. Almost all nucleotide substitutions observed between individuals were found to be silent. The number of nucleotide substitutions between species is presented in Table 2. The number of nucleotide substitutions between *K. pelamis* (SKJ) and *Thunnus* tuna species is large compared with those between species within *Thunnus,* ranging from 35 to 42 nucleotide substitutions (12.0–14.4%) for the 292-bp cytb fragment and from 58 to 69 (14.5–17.3%) for the 400-bp ATP fragment. In the genus *Thunnus,* the dominant type (albacore-like type) of *T. t. orientalis* (PNB) differed from *T. t. thynnus* (ANB) by 7 to 10 nucleotide substitutions (2.4–3.4%) in cytb and by 22 to 23 (5.5–5.8%) in ATP, while it differed from *T. alalunga* (ALB) by only 0 to 3 substitutions (0–1.0%) in cytb and 5 to 7 (1.3–1.8%) in ATP. In both genes, the rare type (Atlantic-like type; designated PNB1) appeared to have nucleotide sequences identical to *T. t. thynnus* (ANB). There were marked similarities among *T. t. thynnus* (ANB), *T.*

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                    1111111111111111111112222222222222222
          1112233366779000122233455566788899001123455667788
          9058140390658325870395810392540382547695195814069 28
ALB3      ACCTCTTATATTCCCCTTCCTCCGCAGCTTCCTCCCTCTGAAACATTGCCT
ALB13     ..........C.....................................
ALB*      ............Y.....................S...........
PNB2,6    ..............................................
PNB3      ..........................................G.....
PNB1      .......G..C..........T........T....A...A.....C.....
ANB*      .......G..C..........T........TY..YA...A....RC.....
SBT9      .....C....C..........T........T....A...A.....C.A...
SBT12,105 ..........C..........T........T....A...A.....C.A...
BET*      .........RC........Y..........T....A...A...A.Y.....
LTT1      .......G..C.........................A...A....TC.....
BFT1      ..........C...T.....................A...A...T.C.....
BFT2      ..........C...T.....................A...A.....C.....
BFT3      ....G.....C...T.....................A...A.....C.....
YFT*      ..........C...........Y.......Y....A..YAG..Y.Y.....
SKJ9      .GTC..C.A..CA..TCCA.A..ATCCTCC.TCTTTCTCC.TCATCA.TTC
SKJ19     .GTC..C.A..CA..TCCA.A..ATCCTCC.TCTTTCTAC.TTATCA.TTC
SKJ20     GGTC..C.A..CA..TCCA.A..ATCCTCC.TCTTTCTAC.TTATCA.TTC
SKJ21     .GTC..C.A..CA..TCCA.A..ATCCTC..TC.TTCTCC.TCATCA.TTC
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Fig. 2. Alignment of 51 variable nucleotide sites observed between individuals in a 292-bp partial nucleotide sequence of the mitochondrial cytochrome *b* gene. See Table 1 for the abbreviated common name. Sequence data of four tuna species with asterisk are cited from Bartlett and Davidson (1991), where R, S, and Y indicate A or G, G or C, and C or T, respectively. Nucleotide sequences of ALB13, BFT1, LTT1, PNB3, SBT12, and SKJ20 are available in DDBJ, EMBL and GenBank Nucleotide Sequence Database under the accession numbers D63491–D63496, respectively.

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                    1111111111111111111111112222222222222222222222222333333333333333333333334
          1222344556677799001112233345556677888999900011222233445566667888801122233344445556677890
          8656844958480361402578470368145340614703492581403692514092589703691462581470369258180361 0
ALB6      TCTCAATAACGCACTCCTGTTGTTGCCCTATCTTTTCGCACACCATAACTTAACTACTTATTTTTCGCGCTTAAGATGATTAAGCCTTC
ALB13     ..................................................T........T.....................C.
ALB14     ..................C.....................................................................
ALB22     ..........................................................................GA......C.....C.
PNB3,4    ................................C.....................C..........C.............T.C.
PNB6      ..................C.............C.....................C..........C.............T.C.
PNB15     ..............T.................C.....................C..........C.............T.C.
PNB1      ..C.C.............A......C...C.C..AT....T.C.CT..G..C..CC......TA.A.C...A..A.CC......C.
ANB36,46  ..C.C.............A......C...C.C..AT....T.C.CT..G..C..CC......TA.A.C...A..A.CC......C.
SBT4      ....C.............A......C...C.C..AT....T.C.CT..G..C..CC....C..A.A.....A..A..C......C.
SBT105    ....C...........C..A......C...C.C..AT....T.C.CT..G..C..CC....C..A.A.....A..A..C......C.
BET5      C...C......T......C..A......C...C.CC..TG...T.C.CT.....C..CAG......A.A.C...A.CA..C......C.
BET6      C...C......T........CA......C...C.CC.ATG...T.C.CT.....C..CAG......A.A.C...A.CA..C......C.
BET10     C...C......T........A......C...C.CC.ATG...T.C.CT.....C..CAG..C...A.A.C...A.CA..C......C.
BET11     C...C......T........A......C...C.CC.ATG...T.C.CT.....C..CAG......A.A.C...A.CA..CG.....C.
LTT1      CTC.C.............A......C...C.C..AT....T.C.CT..G..C..CC.....CTA.ATC...A..A..C......C.
LTT2      CTC.C...........C..A......C...C.C..AT....T.C.CT..G..C..CC.....CTA.ATC...A..A..C......C.
BFT1,2,3  C.C.C...........C..A......C...C.C..AT....T.C.CT.....C..CC......T..ATC...A..A..C......C.
BFT4      C.C.C.........G.C..A......C...C.C..AT....T.C.CT.....C..CC......T..ATC...A..A..C......C.
YFT5      C...C.............A......C...C.CC.AT....T.C.CT..G..C..CC......TA.ATC...A..A..C......C.
YFT6      C...C.....................C...C.CC.AT....T.C.CT..G..C..CC......TA.ATCC..A..A..C......C.
YFT22     C...C...........C..A......C...C.CC.AT....T.C.CT..G..C..CC......TA.ATC...A..A..C......C.
SKJ9      CA.T.TACG.A.CAAT.CCC..CCATTT.TCTCCCCAA.GTCTCTCC.TAAGGACC..A.CA.CC.ATA.C.C.AT.AT..CCAT.C.A
SKJ20     CA.T.TACG.A.CAAT.CCC..CCATTT.TCTCCCCAA.GTCTCTCC.TAAGGACC..A.CA.CC.ATA.C.C.AT.ATC.CCAT.C.A
SKJ21     CA...TACGTA.CAAT.CCC..C.AT.T.TCTCCCCAA.GTTTCTCC.TAAGGACC..A..ACCC.ATA.C.C.AT.A...CCAT.C.A
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Fig. 3. Alignment of 89 variable nucleotide sites observed between individuals in a 400-bp partial nucleotide sequence of the mitochondrial ATPase gene. See Table 1 for the abbreviated common name. Nucleotide sequences of ALB13, ANB36, BET6, BFT1, LTT1, PNB3, SBT4, SKJ20, and YFT5 are available in DDBJ, EMBL and GenBank under the accession numbers D63414–D63422, respectively.

maccoyii (SBT), and species of the yellowfin group. The number of nucleotide substitutions between *T. t. thynnus* (ANB) and *T. maccoyii* (SBT) ranged from 2 to 5 (0.7–1.7%) in cytb and from 5 to 6 (1.3–1.5%) in ATP, and those between these bluefin tunas and the tropical species of *T. albacares* (YFT), *T. atlanticus* (BFT), and *T. tonggol* (LTT) ranged from 3 to 8 (1.1–2.7%) in cytb and 4 to 10 (1.0–2.5%) in ATP. Tropical species appeared to have mtDNA sequences very similar to one another, where the nucleotide substitutions between these species ranged from 2 to 5 (0.7–1.7%) in cytb and 3 to 7 (1.1–1.8%) in ATP. *T. obesus* (BET) differs from *T. alalunga* (ALB) and *T. t. orientalis* (PNB) by 5 to 8 nucleotide substitutions (1.7–2.7%) in cytb and 24 to 31 (6.0–7.8%) in ATP. Deviation of *T. obesus* (BET) from *T. t. thynnus* (ANB), *T. maccoyii* (SBT), and species of the yellowfin group is much smaller: the number of nucleotide substitutions between *T. obesus* (BET) and the others ranged from 3 to 7 (1.1–1.8%) in cytb and 10 to 14 (2.5–3.5%) in ATP.

Table 2. Number of nucleotide substitutions between species in cytb (above diagonal) and ATP (below diagonal)

Species	ALB	PNB	ANB	SBT	BET	LTT	BFT	YFT	SKJ
ALB		0–3	7–10	7–9	6–8	6–7	5–7	5–8	35–39
PNB[a]	5–7		7–10	7–9	5–7	6–7	5–7	5–8	35–38
ANB	23–26	22–23		2–5	3–7	3–5	4–7	4–8	38–40
SBT	20–24	21–23	5–6		3–6	5–6	4–6	4–7	39–42
BET	25–29	24–31	12–13	11–12		4–6	3–6	3–5	35–40
LTT	25–30	25–27	5–6	8–10	13–14		3–4	3–5	35–38
BFT	25–29	23–26	6–7	8–10	12–13	4–6		2–5	36–40
YFT	25–27	22–24	4–6	4–7	10–13	3–6	3–7		35–39
SKJ	62–69	61–66	62–65	59–63	58–64	59–64	62–67	60–64	

[a] PNB1 was not included

Since tree topologies obtained from cytb and ATP data and those by NJ and ML methods for phylogenetic analysis were almost consensus, only the result of the NJ method results using the ATP data are shown in Fig. 4. In the genus *Thunnus* two distinct clades are evident: one consists of *T. alalunga* (ALB) and the dominant type of *T. t. orientalis* (PNB), and the other consists of the other *Thunnus* species including the rare type of *T. t. orientalis* (PNB1). Bootstrapping (1,000 replications) indicated that the separation between these two clades and among all species is highly significant. The resulting tree topology by the ML method was compared with the best tree among those pairing *T. t. thynnus* (ANB) and *T. t. orientalis* (PNB). The log likelihood ratio was 72.7 with a standard error of 20.7, and the latter tree was rejected as not significant.

RFLP Analysis on ITS1 Fragment

RFLPs between species were detected in all endonuclease digestions, whereas no RFLP between individuals within species was observed among three to six individuals examined in each species. Representative RFLP between species obtained by *Fnu*4HI digestion is shown in Fig. 5. The fragment distributions in each enzyme digestion of all species are presented in Table 3. There were no differences in the restriction patterns of all endonuclease digestions between *T. t. thynnus* (ANB) and *T. t. orientalis* (PNB), between *T. obesus* (BET) and *T. maccoyii* (SBT), and between *T. atlanticus* (BFT) and *T. albacares* (YFT) tunas. *T. alalunga* (ALB) was found to share the least number of fragments with the other species (45.5–65.2%), followed by *T. t. thynnus* and *T. t. orientalis* (ANB and PNB) (63.6–69.8%). The other species were observed to share a larger number of fragments with one another (85.7–100%).

Discussion

The present results on mtDNA and nDNA are consistent with allozyme analysis by Sharp and Pirages (1978) and Elliott and Ward (1995) for *T. alalunga* (ALB), which showed it to be the most divergent member of the genus. Although mtDNA of *T. t. orientalis* (PNB) is very similar to that of *T. alalunga* (ALB), the allozyme analysis revealed *T. t. orientalis* (PNB) to have closest affinity to *T. t. thynnus* (ANB), *T. maccoyii* (SBT), and even to the species of yellowfin tuna group. Our preliminarly allozyme analysis also indicated *T. alalunga* (ALB) to have fixed allelic differences from the bluefin tunas (ANB, PNB, and SBT) at the aspartate aminotransferase, phosphoglucomutase, and peptidase loci, while we failed to find fixed differences between these bluefin tunas. RFLP analysis on PCR-amplified DNA fragments of ITS1 fragment also indicated deviation of *T. alalunga* (ALB) from the other species and closest relationships between *T. t. thynnus* (ANB) and *T. t. orientalis* (PNB). Thus, the Atlantic and Pacific northern bluefin tunas (*T. t. thynnus* and *T. t. orientalis*) are very different at mtDNA but almost identical at nDNA. Strong isolation appears to have acted to maintain the large mtDNA differentiation between these two populations of the northern bluefin tuna. However, it is highly improbable that the mtDNA lineages of *T. t. orientalis* (PNB) independently came to share similar sequences with *T. alalunga* (ALB). The best explanation for the sequence similarity between *T. alalunga* (ALB) and *T. t. orientalis* (PNB) is interspecific transfer of mtDNA, with *T. alalunga* (ALB) mtDNA introduced by hybridization having replaced most of the original mitochondrial lineages in the Pacific population of the northern bluefin tuna (PNB). On the other hand, nDNA introduced from *T. alalunga* (ALB) must have largely been lost in backcross generations. To satisfy these scenarios, the population size of *T. t. orientalis* (PNB) may had been considerably smaller than that of *T. alalunga* (ALB) as the hybridizations preferentially take place between parental species differing greatly in abundance (Avise and Saunders 1984). Further, there must be barrier against introgression of *T. alalunga* (ALB) nDNA in the subsequent backcross generations under little or no selection acting against *T. alalunga* (ALB) mtDNA, as suggested in the case of *Drosophila* (Powell 1983). The introgression could be initiated by a single or only a few hybridization events between a limited number of *T. t. orientalis* (PNB) males

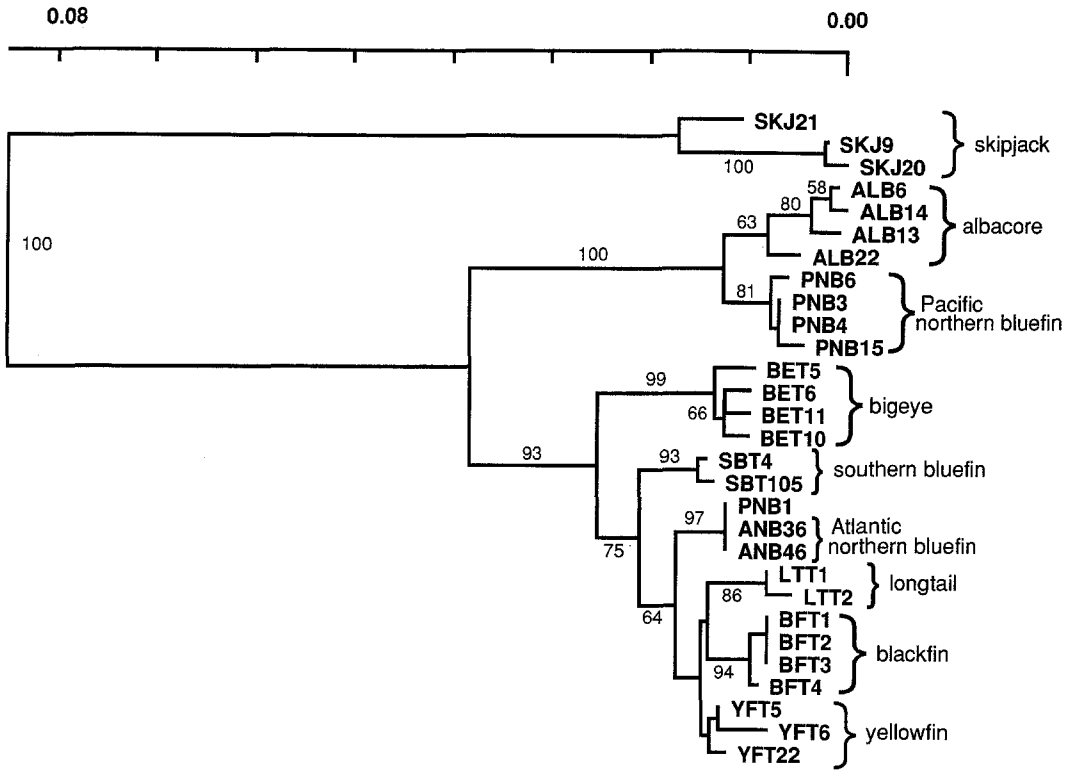

Fig. 4. Phylogenetic tree constructed using the neighbor-joining (NJ) method based on genetic distances estimated from the mitochondrial ATPase gene sequences of 26 individuals of the genus *Thunnus* and three individuals of *Katsuwonus pelamis* (SKJ) as an outgroup. Bootstrap values larger than 50% are shown on branches.

and *T. alalunga* (ALB) females, and in the subsequent backcross generations constrained breeding between the hybrid stem and *T. t. orientalis* (PNB) is highly necessary. Atlantic-like type (PNB1) observed may be a descendant of migrants from the Atlantic. Even so, the migrants must have been very small in number, as indicated by the distribution (Collette and Nauen 1983), and also very recent, as it is unlikely that this mtDNA lineage would have been static without accumulating a certain number of nucleotide substitutions. Similar mtDNA introgression across a species boundary is observed in several terrestrial animals (Ferris et al. 1983; Powell 1983; Spolsky and Uzzell 1984). Although gene introgression seems to be more common in fish than in the other vertebrates (Smith 1992), the present study is the first in which molecular evidence for introgressive hybridization in marine pelagic species is reported. Mitochondrial gene introgression between fish species does not seem to be very unusual, especially among species at lower taxonomic levels where the likelihood of hybridization may not be proportional to genetic similarity (Rosen 1978; Hillis 1988). Use of mtDNA for phylogenetic analysis should proceed with caution, especially for closely related species, and supplementary analysis of nuclear genomes is strongly recommended.

Assuming a divergence rate of 2–4% per 1×10^6 years for mtDNA (Brown et al. 1982), we may roughly esti-

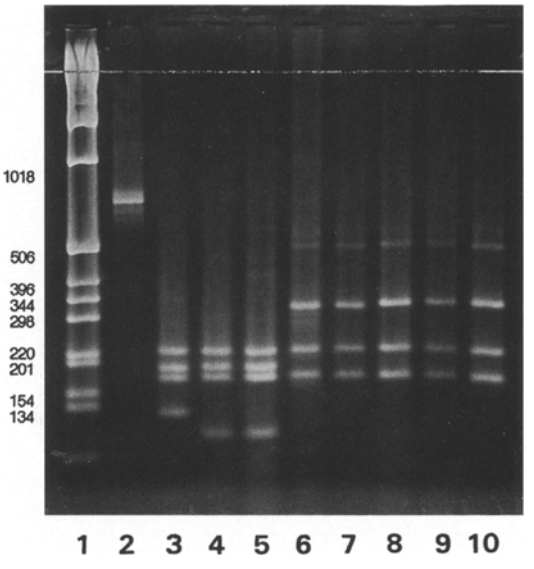

Fig. 5. EtBr-stained ITS1 fragments of all *Thunnus* tuna species digested by *Fnu* 4HI. *Lanes 1:* 1-kb DNA ladder (sizes in base pairs are shown at the *left*); *2:* undigested fragment; *3: T. alalunga* (ALB); *4: T. thynnus orientalis* (PNB); *5: T. t. thynnus* (ANB); *6: T. maccoyii* (SBT); *7: T. obesus* (BET); *8: T. tonggol* (LTT); *9: T. atlanticus* (BFT); and *10: T. albacares* (YFT).

Table 3. ITS1 fragment distributions of tuna species digested by eight endonucleases[a]

Fragment size in base pair	ALB	PNB	ANB	SBT	BET	LTT	BFT	YFT
*Bsa*JI								
155	1	0	0	1	1	1	1	1
130	0	1	1	0	0	0	0	0
125	1	1	1	1	1	1	1	1
110	1	1	1	0	0	0	0	0
80	1	1	1	0	0	0	0	0
70	1	1	1	1	1	1	1	1
*Dde*I								
350	1	0	0	0	0	0	0	0
340	0	1	1	1	1	1	1	1
240	0	0	0	1	1	1	1	1
235	0	1	1	0	0	0	0	0
230	1	0	0	0	0	0	0	0
115	1	1	1	1	1	1	1	1
*Fnu*4HI								
300	0	0	0	1	1	1	1	1
220	1	1	1	1	1	1	1	1
195	1	1	1	0	0	0	0	0
180	1	1	1	1	1	1	1	1
130	1	0	0	0	0	0	0	0
115	0	1	1	0	0	0	0	0
*Hae*III								
275	1	1	1	0	0	0	0	0
230	0	0	0	1	1	1	1	1
110	1	1	1	1	1	1	1	1
100	1	1	1	1	1	1	1	1
*Mbo*I								
700	0	1	1	1	1	1	1	1
360	1	0	0	0	0	0	0	0
295	1	0	0	0	0	0	0	0
*Mse*I								
700	1	0	0	0	0	0	0	0
440	0	1	1	1	1	1	1	1
260	0	1	1	1	1	1	1	1
*Sau*96I								
155	0	1	1	1	1	1	1	1
140	1	0	0	0	0	0	0	0
125	1	1	1	1	1	1	1	1
115	1	1	1	1	1	1	1	1
80	0	0	0	0	0	0	1	1
75	0	0	0	1	1	1	0	0
70	1	1	1	0	0	0	1	1
*Taq*I								
700	1	1	1	1	1	0	0	0
360	0	0	0	0	0	1	1	1
340	0	0	0	0	0	1	1	1

[a] Fragment present (1) and absent (0)

mate the time of speciation. *T. alalunga* (ALB) appears to be the earliest offshoot in the genus, having a common ancestor ~2 × 10^6 years ago. *T. obesus* (BET) subsequently diverged from the stem ~1 × 10^6 years ago. Bluefin tunas (ANB and SBT) and species of the yellowfin group (BFT, LTT, and YFT) showing less mtDNA divergence from one another may have emerged ~500,000 years ago. Elliott and Ward (1995) indicated that the *Thunnus* tuna species appear to be genetically much more similar one another than are most congeneric fish species and pointed out that the relationships between bluefin tunas (PNB and SBT) and *T. albacares* (YFT) are too close to infer the branch orders from their allozyme data. MtDNA divergence between *T. t. orientalis* (PNB) and *T. alalunga* (ALB) is comparable with those among *T. t. thynnus* (ANB), *T. maccoyii* (SBT), and species of the yellowfin group, suggesting an introgression event to have occurred at similar timing with the emergence of the bluefin tunas and tropical tuna species. Thus, molecular data of mitochondrial and nuclear genomes are consistent with one another in consideration of mtDNA transfer between *T. alalunga* (ALB) and *T. t. orientalis* (PNB). Molecular data presented in this study as well as in several previous studies (Sharp and Pirages 1978; Chow and Inoue 1993; Block et al. 1993; Finnerty and Block 1995; Elliott and Ward 1995) present a considerable deviation from the phylogenetic implication based on the morphology, indicating that reevaluation of the morphological characteristics may be necessary.

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