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Genetic identification of *Thunnus orientalis, T. thynnus*, and *T. maccoyii* by a cytochrome *b* gene analysis

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Abstract The three species of bluefin tunas, *Thunnus orientalis*, *T. maccoyii*, and *T. thynnus,* are morphologically similar, which can pose problems for fisheries management and marketing. We examined intraspecific genetic diversity and interspecific genetic boundaries among these three species by analyzing the cytochrome (Cyt) *b* gene. The full lengths of the nucleotide sequences were 1,141 bp in *T. orientalis* and *T. thynnus* and ranged 1,138~1,141 bp in *T. maccoyii*. Mean nucleotide diversities were 0.0019± 0.0002 in *T. thynnus* (n=8), 0.0063±0.0005 in *T. orientalis* (n=22), and 0.0059±0.0007 in *T. maccoyii* (n=24). Average numbers of nucleotide differences and nucleotide substitutions per site among the three species were 18.748±2.879 and 0.017±0.003, respectively. The Neighbor-joining and minimum-evolution trees showed distinct clades with high bootstrapping value support, and the high *F*st value indicated significant differentiation among the three species. *T. thynnus*, *T. orientalis,* and *T. maccoyii* could be individually distinguished from each other *Thunnus* tunas by the 132nd, 375th, and 1,023rd sites of the Cyt *b* sequences. In the mismatch analysis, Fu's and Tajima's tests of sequences from *T. orientalis* and *T. maccoyii* provided evidence of their population expansion dating to the middle Pleistocene.

Keywords Middle Pleistocene · Nucleotide diversity · Population expansion · Tuna

Introduction

Thunnus South, 1845 contains eight species which are important commercial and recreational fisheries throughout the tropical and temperate oceans of the world. *Thunnus thynnus* (Linnaeus, 1758), *T. maccoyii* (Castelnau, 1872), and *T. orientalis* (Temminck and Schlegel, 1844) are bluefin tunas which are morphologically similar but have different geographic distributions (Collette et al. 2001). In the West Atlantic, *T. thynnus* is found from Brazil to Newfoundland (Porch 2005); in the East Atlantic, it is found from roughly the Canary Islands to south of Iceland, and throughout the Mediterranean Sea. *Thunnus maccoyii* is solely distributed in temperate and cold areas of the Indian Ocean between 30°S and 50°S, and to nearly 60°S and in the southwestern Pacific Ocean around Australia and New Zealand (Caton 1991). *Thunnus orientalis* is primarily found in the North Pacific i.e., the Gulf of Alaska to southern California, Baja

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California, and the area from Sakhalin Island in the southern Sea of Okhotsk to the northern Philippines (Bayliff 2001). It was also caught in the Gulf of Papua, New Guinea (Collette and Smith 1981) and is occasionally caught in Australia, the Galapagos Islands, and New Zealand (Bayliff 1994; Ward et al. 1995; Smith et al. 2001).

Atlantic bluefin tuna has an entirely different distribution from other two species. Gibbs and Collette (1967) described how the anatomical characters of Atlantic and Pacific bluefin tunas are extremely similar and suggested that they should be defined as subspecies. Subsequently, Collette (1999) indicated that the Pacific bluefin is a full species based on genetic and morphological data. Even though the habitats of the two species of Pacific and Southern bluefin tunas partially overlap, they can generally be distinguished by the position of the first ventrally directed parapophysis, i.e., on the 9th vertebra in Southern bluefin but on the 8th vertebra in Pacific bluefin, and the yellow caudal keel in Southern bluefin vs. a dark one in Pacific bluefin. However, distinguishing the Pacific bluefin from the Southern bluefin based on the color of the caudal keel alone is insufficient since this superficial character is not stable (Collette and Nauen 1983, Anonymous 1994, Smith et al. 1994). As mentioned above, these three bluefin tunas are morphologically similar and may be misidentified, posing problems for fisheries management and marketing.

Molecular techniques have long been used to identify fish specimens and explore the population structure of tuna (Ward et al. 1997; Alvarado-Bremer et al. 1998; Chow et al. 2000; Takeyama et al. 2001; Bottero et al. 2007). Protein electrophoresis and restriction fragment length polymorphism (RFLP) of mitochondrial (mt)DNA are the most commonly used methods in published reports. Recently, mtDNA sequences, especially the cytochrome (Cyt) *b* gene, are frequently utilized to study species identification and historical demography. For example, Tseng et al. (2009) successfully distinguished two morphologically similar *Acanthopagrus* species using the Cyt *b* gene, and some similar methodologies were also published by Akimoto et al. (2006). Ward et al. (1995) reported one diagnostic locus (s*AH**) and two nearly diagnostic loci (*ADA** and *GDA**) from an allozyme analysis and species-diagnostic restriction digest profiles used to explore genetic differences between Pacific and Southern bluefin tunas. Their conclusions indicated that an mtDNA analysis can provide more-reliable discrimination of specimens than would an allozyme analysis.

In addition, exploring the genetic diversity of species will help elucidate their historical population dynamics (Zhao et al. 2008). The patterns and amounts of diversity in DNA sequences are informative in inferring the history of a population as well as the mechanisms responsible for generating and maintaining polymorphisms (Li 1997). In other words, dramatic changes in the environment may leave imprints in the gene pool of a population.

In the Pleistocene, periodic climatic oscillations greatly changed species' geographical distributions and abundances, which likely influenced the genetic diversity of many species (Avise 2000; Hewitt 2000). The development of methods to test mutation-drift equilibrium (MDE) allows the historical demography of a population to be traced using mtDNA data. Mismatch distributions, Fu's *F*s, and Tajima's *D* tests were proposed to resolve the population history (Tajima 1989; Fu 1997; Schneider and Excoffier 1999; Wu et al. 2009). The Indian and Pacific Oceans appear to provide great natural settings to study historical dynamics of marine species due to the great abundance of species diversity in these oceans.

The main objective of this study was to use molecular characters of the mtDNA Cyt *b* gene to discriminate among *T. thynnus*, *T. orientalis*, and *T. maccoyii* and estimate interspecific and intraspecific genetic diversity. Moreover, we also explored the historical demography of *T. orientalis* and *T. maccoyii*.

Materials and methods

Sampling

Twenty-two *T. orientalis* individuals were caught from the northwestern Pacific Ocean (26°N, 121°45"E) in 2007 and their tissue samples were collected from the Tongkung fish market, southwestern Taiwan. Twenty-four *T. maccoyii* individuals were obtained from the Indian Ocean by Taiwanese fisheries observers in 2006 and 2007 as described by Shiao et al. (2008, 2009). Species were primarily identified by the color of the caudal keel and sampling location (Collette and Nauen 1983). Muscle tissues were preserved in 95% ethanol until the DNA was extracted.

DNA isolation and polymerase chain reaction (PCR) amplification

Crude DNA was extracted from 500-mg samples of skeletal muscle using the method of Kocher et al. (1989). The complete Cyt *b* gene was amplified using the forward primer, 5'-ACCAGGACTAATGGCTTG-3', and reverse primer, 5'-AGGATTTTAACCTCCG ACGTC-3', which were developed in this study by referring to the complete mtDNA sequence of *T. thynnus* (Manchado et al. 2004). A PCR consisted of approximately 5 ng genomic DNA, 50 pmol each of the forward and reverse primers, 25 mM dNTP, 0.05~ 0.1 mM $MgCl_2$, 10× buffer, and 5 U *Taq* polymerase (Takara Shuzo, Shiga, Japan) brought to 125 μl with Milli-Q water. The PCR program included one cycle of 5 min at 95°C; and 38 cycles of 1 min at 95°C, 1 min at 54°C, and 1.5 min at 72°C; followed by a single further extension of 10 min at 72°C. We evaluated 10 μl of each product on a 0.8% agarose gel to check the PCR success and confirm the product sizes. The remaining PCR products were run on 0.8% agarose gels and purified using a DNA Clean/ Extraction kit (GeneMark, Taichung, Taiwan). Purified DNA was subcloned into the pGEM-T easy vector (Promega, Madison, WI, USA) and transformed into the *Escherichia coli* JM109 strain. Plasmid DNA was isolated using a mini plasmid kit (Geneaid, Taichung, Taiwan). Clones from 22 and 24 individuals of *T. orientalis* and *T. maccoyii*, respectively, were sequenced on an Applied Biosystems (ABI, Foster City, CA, USA) automated DNA sequencer 377 (vers. 3.3) using a Bigdye sequencing kit (Perkin-Elmer, Wellesley, MA, USA). The T7 and SP6 primers were used in the sequencing reaction, and the PCR cycle parameters for sequencing were 35 cycles of 30 s at 95°C, 30 s at 50°C, and 1 min at 72°C.

Cyt *b* gene analysis

In total, 46 Cyt *b* sequences subcloned from *T. orientalis* and *T. maccoyii* were deposited in GenBank (accession nos.: *T. orientalis*, AM989952~73 and *T. maccoyii*, AM989928~51). Eight homologous sequences of *T. thynnus* derived from NCBI (NC_004901, NC_014052, EF427612.1, EF439243.1, EF439609.1, EU036522.1, AY302574.2, and AB097669.1) were selected as reference sequences in this study. All sequences were aligned using Clustal W (Thompson et al. 1994) and then checked with the naked eye. Different nucleotide ratios within the 54 sequences were estimated. Intraspecific and interspecific genetic diversities of nucleotides were calculated using the MEGA software (Tamura et al. 2007). The general time-reversible plus invariant sites plus gamma distributed model (GTR+I+G) is the best-fitting model for DNA substitutions as determined by the Modeltest 3.7 program (Posada and Crandall 1998) using the Akaike information criterion (AIC=5370.45). The phylogenetic tree of nucleotide sequences was constructed using Neighbor-joining (NJ) and minimum-evolution (ME) methods with an interior-branch test (Nei and Kumar 2000). The confidence of the clusters was assessed using a bootstrap analysis with 1,000 replications. The minimum spanning tree (MST) was computed from the matrix of pairwise distances between all pairs of haplotypes in each sample using a modification of the algorithm described by Rohlf (1973). Amino acid sequence divergence was estimated using Poisson correction methods (Nei and Kumar 2000) in MEGA (Tamura et al. 2007). The phylogenetic tree of amino acid sequences was constructed by Neighbor-joining method with an anterior-branch test, and the confidence of the clusters was assessed by 1,000 bootstrap replications.

We evaluated whether sequences had evolved under strict neutrality. Fu's *F*s (Fu 1997) and Tajima's neutrality tests (Tajima 1989) were performed in Arlequin 3.1 (Excoffier et al. 2005). The significance of the statistics was tested by generating random samples under the hypothesis of selective neutrality and population equilibrium, using a coalescent simulation algorithm adapted from Hudson (1990). Tajima's test is based on an infinite-site model without recombinations. A significant *D* value can be due to factors other than selective effects, like population expansion, a bottleneck, or heterogeneity of mutation rates (Tajima 1996).

The possible occurrence of historical demographic expansions was examined using the mismatch distribution (Schneider and Excoffier 1999) implemented in Arlequin (Excoffier et al. 2005). The distribution is unimodal in samples following a population demographic expansion (Rogers and Harpending 1992; Rogers 1995). The parameter of demographic expansion, τ, is estimated by a generalized nonlinear least-squares approach, and confidence intervals of the parameter are computed using a parametric bootstrap

approach (Schneider and Excoffier 1999). τ is an index of time since expansion and is expressed in units of mutational time (Slatkin and Hudson 1991). We transformed the value of τ to an estimate of time since expansion using the equation, $\tau=2ut$, where u is the mutation rate per locus per generation and t is the number of generations since the expansion. The value of u is derived from $u=\mu k$, where μ is the mutation rate per nucleotide per generation, and k is the number of nucleotides in the sequence. The significance of the estimate was obtained by calculating the sum of the squared deviation (SSD) statistic and the raggedness statistic (Harpending 1994). For each statistic, the sudden expansion model was rejected when $p<0.05$ (Excoffier et al. 2005).

Results

The total length of the Cyt *b* gene was 1,138~1,141 bp among specimens of *T. maccoyii* and 1,141 bp among specimens of *T. orientalis* and *T. thynnus*. The percentages of the nucleotide composition did not significantly differ among the three bluefin tuna species. Altogether seven polymorphic sites occurred within eight sequences of *T. thynnus*. Values of H_d, k, and Pi within these sequences were 1, 2.214, and 0.0019±0.0002. In total, 51 polymorphic sites and 11 parsimoniously informative sites occurred within the 22 sequences of *T. orientalis* (Fig. 1). Haplotype diversity (H_d), the average number of nucleotide differences (k), and the nucleotide diversity (Pi) within these sequences were 1, 7.528, and 0.0066±0.0004, respectively. Forty-eight polymorphic sites and eight parsimoniously informative sites occurred within the 24 sequences of *T. maccoyii*. All sequences of *T. maccoyii* in this study were diverse except for one identical haplotype found in three specimens (S01, S03, and S05). Values of H_d, k, and Pi within the 24 sequences of *T. maccoyii* were 0.917, 6.706, and 0.0059±0.0007, respectively (Table 1). There were 28 fixed differences within specimens of *T. maccoyii*. Six mutations were common between *T. orientalis* and *T. maccoyii* (Fig. 1). The number of nucleotide substitutions ranged 2~13 among haplotypes of *T. orientalis*, 1~15 among haplotypes of *T. maccoyii*, 1~3 among haplotypes of *T. thynnus*, and 8~47 among the three species. The k value and average number of nucleotide substitutions per site (D_{xy}) between *T. orientalis* and *T. maccoyii* were 40 and 0.036±0.0023, respectively. Base pair 831 had different compositions in the three bluefin tuna: it was thymidine in Pacific bluefin, guanine in southern bluefin, and adenine in Atlantic bluefin. The base pair 32 guanine was solely present in *T. thynnus* but not in the other two *Thunnus* species.

Intraspecific Kimura-2-parameter genetic distances among different haplotypes ranged 0.001~0.020 for *T. maccoyii*, 0.002~0.012 for *T. orientalis*, and 0.001~0.003 in *T. thynnus*. The interspecific distances ranged from 0.007 (*T. maccoyii* vs. *T. thynnus*) to 0.043 (*T. maccoyii* vs. *T. orientalis*) among the three bluefin tuna species (Table 2). The mean nucleotide diversity among the three species ranged from 0.009±0.002 (*T. maccoyii* vs. *T. thynnus*) to 0.037±0.005 (*T. maccoyii* vs. *T. orientalis*). The coefficients of differentiation among the three species were 0.706±0.04 (*T. orientalis* vs. *T. maccoyii*), 0.742±0.04 (*T. orientalis* vs. *T. thynnus*), and 0.436±0.07 (*T. maccoyii* vs. *T. thynnus*). Specimens of *T. orientalis*, *T. thynnus*, and *T. maccoyii* were distributed in three monophyletic clades in the NJ and ME phylogenetic tree, with 99%, 98%, and 81% bootstrap support, respectively (Fig. 2). Atlantic bluefin tuna and southern bluefin tuna were shown to be sister taxa.

The Cyt *b* gene is highly conserved and codes a protein of 380 amino acids. After alignment, four identical amino acid sequences were observed in four specimens of *T. orientalis* and 11 specimens of *T. maccoyii*. Eight sequences of *T. thynnus* from NCBI's GenBank coded an identical amino acid sequence. In total, 39 polymorphic amino acid sites were found within 36 different sequences from the three bluefin tuna species (Fig. 3). The intraspecific Poisson correction distances of different amino acid sequences within *T. orientalis* as well as in *T. maccoyii* ranged 0.003~0.016. Interspecific genetic distances among the three bluefin tuna species ranged 0.003 (*T. maccoyii* vs. *T. thynnus*) ~0.021 (*T. maccoyii* vs. *T. orientalis*) (Table 2). Mean distances within specimens of *T. orientalis* and *T. maccoyii* were 0.008±0.002 and 0.007±0.002, respectively. Interspecific mean distances ranged from 0.007±0.003 (*T. maccoyii* vs. *T. thynnus*) to 0.013±0.004 (*T. orientalis* vs. *T. maccoyii*). Most of the nucleotide substitutions observed between sequences were silent mutations. However, the 125th and 341st sites of the amino acid sequence had specific compositions among *T. orientalis* (Met and Ile), *T. maccoyii* (Ile and Met), and *T.*

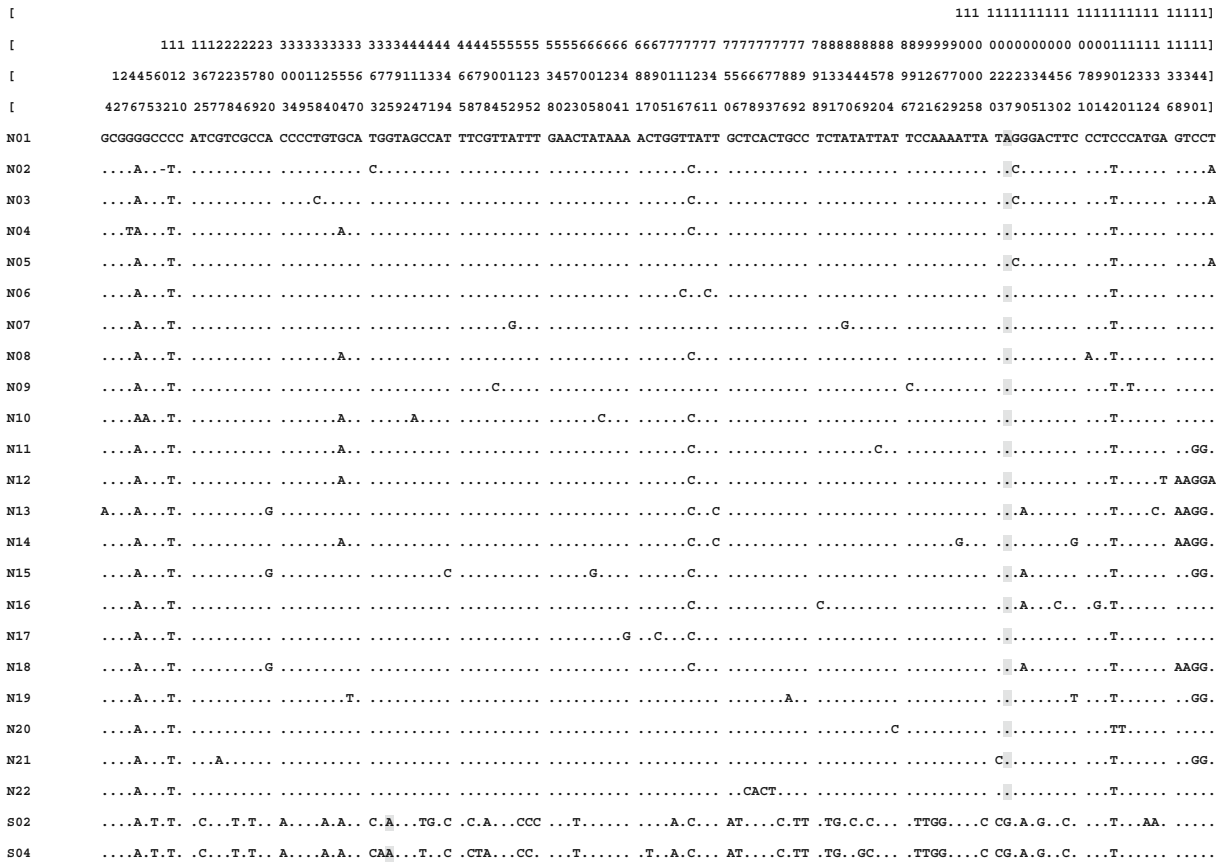

Fig. 1 Variable sites at aligned nucleotide sequences of the cytochrome *b* gene for 46 specimens of *Thunnus orientalis* (N) and *T. maccoyii* (S). The eight sequences of *T. thynnus* were obtained from NCBI's GenBank

thymus (Met and Met). In total, 22 and 17 private amino acid variations were present in *T. orientalis* and *T. maccoyii*, respectively. Only one common variation occurred at the 380th position (Fig. 3). The NJ topology of amino acid sequences indicated a failure to resolve interrelationships among these species (Fig. 4). *Thunnus thynnus* has a closer relationship to *T. maccoyii* than to *T. orientalis* based on the data of nucleotide sequences, but the reverse situation occurred in the analytic results from the amino acid sequences.

Mismatch analyses of the Cyt *b* data indicated a recent population expansion in both species. Significant ($p<0.02$ and $p=0$) negative values of Tajima's D (-1.824 and -1.982) and Fu's F_s (-17.664 and -15.286) were present in *T. orientalis* and *T. maccoyii*, respectively. In particular, the F_s value is very sensitive to recent population demographic expansions, which generally lead to large negative values. A plot of the mismatch distribution showed that the mean numbers of pairwise differences were 3.761 for *T. orientalis* and 3.912 for *T. maccoyii,* and showed that they were unimodal (Fig. 5), indicating an expanding population. The sudden expansion model parameters in *T. orientalis* were $\tau=7.496$ (97.5% confidence limits (CLs): 4.941~8.965) and in *T. maccoyii* were $\tau=5.273$ (97.5% CLs: 1.664~14.316). The SSDs between the observed and expected mismatch and raggedness index were 0.0042 ($p=0.52$) and 0.0175 ($p=0.35$) in *T. orientalis*; and were 0.0055 ($p=0.60$) and 0.0092 ($p=0.92$), in *T. maccoyii*, respectively. All data indicated that the sudden expansion model could not be rejected. The MSTs of *T. orientalis* and *T. maccoyii* were characterized by a reticulate phylogeny (Fig. 6). Most of haplotypes were located at the tips.

The generation time estimated for *T. orientalis* was roughly 5 years and for *T. maccoyii* was roughly 10~

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S01_S03_S05 ....A.T.T. .C...T.T.. A....A.A.. C.A...T..C .CTA...CC. ...T...... ....A.C... AT....C.TT .TG..GC... .TTGG....C CG.A.G..C. ...T...... .....
S06         .A..A.T.T. .C...T.T.. G....A.A.. C.A...T..C .C.A...CC. ...T..C.... ...AA.C... A.....C.TT .TG...C... .TTGG....C CG.A.GT.C. ...T..G... .....
S07         ....A.T.T. .C...T.T.. A....A.A.. C.A...T..C .CTA...CC. ...T...... ....A.C... AT....C.TT .TG..GC... .TTGG....C CG.A.G..C. ..CT...... .....
S08         ....A.T.T. .C..CT.T.. A....A.A.. C.A...T..C .C.A...CC. ...T...... ....A.C... AT....C.TT .TG...C... .TTGG....C CG.A.G..C. ...T...... .....
S09         ....A.T.T. .C...TAT.. A....A.A.. C.A...T..C .C.A...CC. ...T...... ....A.C... AT....C.TT .TG...C... .TTGG....C CG.A.G..C. ...T...... .....
S10         ....A.T.T. .C...T.T.. A....A.A.. C.A...T..C .C.A...CC. ...T...... ....A.C... AT....C.TT .TG...C... .TTGG....C CG.A.G..C. ...T...... .....
S11         .A..A.T.T. .C...T.T.. A....A.A.. C.A...T.TC .C.A...CC. ..GT...... ....A.C... A.....C.TT .TG...C... .TTGG....C CG.A.G..C. ...T...... ..GG.
S12         ....A.T.T. .C...T.T.. A....A.A.. C.A...T..C .CTA...CC. ...T...... ....A.C... AT....C.TT .TG..GC... .TTGG....C CG.A.G..C. ...T...... ..GG.
S13         .A..A.T.T. .C...T.T.. AGT..AGA.. C.A.C.T..C .C.A...CC. ...T...... ....A.CG.. A.....C.TT .TG...C... .TTGG....C CG.A.G..C. ...T...... ...G.
S14         ....A.T.T. .C...T.T.. A....A.A.. C.A...T..C .CTA...CC. .G.T...... G...A.C... AT....C.TT .TG..GC... .TTG.....C CG.A.G..C. ...T...... .....
S15         ....A.T.T. .C...T.T.. A..T.A.A.. C.A...T..C .C.A...CC. ...T...... ..G.A.C... A.....C.TT .TG...C... .TTGGG.C.C CG.A.G..C. ...T...... ..GGA
S16         ....A.T.T. .C...T.T.. A....A.A.. C.A...T..C .C.A...CC. ...T...... ....A.C... AT....C.TT .TG...C... .TTGG...CC CG.A.G..C. ...T...... ..GG.
S17         ....A.T.T. .C...T.T.. A....A.A.. C.A...T..C .C.A...CC. ...T....G. ....A.C... AT....C.TT .TG...C... .TTGG....C CG.A.G..C. ...T...... .....
S18         ....A.T.T. .C...T.T.. A....A.A.. C.A...T..C .CTA...CC. ...TC..... ....A.C... AT....C.TT .TG..GC... .TTGG....C CG.A.G..C. ...T...... ..GG.
S19         ..A.A.T.T. .CT..T.T.. A....A.A.. C.A...T..C .CTA.C.CC. ...T...... ....A.C... AT....C.TT .TG..GC... .TTGG....C CG.A.G..C. ...T...... ..GG.
S20         .A..A.T.T. .C...T.T.. A....A.A.. C.A...T..C .C.A...CC. A.GT...... ....A.C... A.....C.TT .TG...C... .TTGG....C CG.ACG..C. ...T...... ..GG.
S21         ....A.T.T. .C...T.T.. A....A.A.. C.A...T..C CC.A...CC. ...T...... ....A.C... AT....C.TT .TG...C.G. .TTGG....C CG.A.G..C. ...T...... ...G.
S22         ....A.T.T. .C...T.T.. A....A.A.. C.A...T..C .C.A...CC. ...T...... ....A.C... AT..G.C.TT .TG...C... .TTGG....C CG.A.G..C. ...T...... .....
S23         ....A.T.T. .C...T.T.. A....A.A.. C.A...T..C .CTA...CC. ...T...G.. ....A.C... AT....C.TT .TG..GC... .TTGG....C CG.A.G..C. ...T...... ..GG.
S24         ....A.T.T. .C...T.T.. A....A.A.. C.A...T..C .CTA...CC. .G.T...... ....A.C... AT....C.TT .TG..GC... .TTGG....C CG.A.G..C. ...T...... ..GG.
AB097669.1  ....A.T.T. GC...T.T.. A....A.A.. C.....T..C .CTA...CC. ..TT...... ....A.C... AT....C.TT .TA..GC... .TTG.....C .G.A.G..C. ...T...... .....
AY302574.2  ....A.TTT. GC...T.T.. A....A.A.G C.....T..C .CTA...CC. ...T...... ....A.C... AT....C.TT .TA..GC... .TTG.....C .G.A.G..C. ...T...... .....
EF427612.1  ....A.T.T. GC...T.TT. A....A.A.G C.....T..C .CTA...CC. ...T...... ....A.C... AT....C.TT .TA..GC... .TTG.....C .G.A.G..C. ...T...... .....
EF439243.1  ....A.T.TT GC...T.T.. A....A.A.. C.....T..C .CTA...CC. ...T...... ....A.C... AT....C.TT .TA..GC... .TTG.....C .G.A.G..C. ...T...... .....
EF439609.1  ....A.T.T. GC...T.T.. A....A.A.. C.....T..C .CTA...CC. ...T...... ....A.C... AT....C.TT .TA..GC... .TTG.....C .G.A.G..C. ...T...... .....
EU036522.1  ....A.T.T. GC...T.T.. A....A.A.. C..C..T..C .CTA...CC. ...T...... ....A.C... AT....C.TT .TA..GC... .TTG.....C .G.A.G..C. ...T...... .....
NC_004901   ....A.TTT. GC...T.T.. A....A.A.G C.....T..C .CTA...CC. ...T...... ....A.C... AT....C.TT .TA..GC... .TTG.....C .G.A.G..C. ...T...... .....
NC_014052   ....A.T.T. GC...T.T.. A....A.A.. C.....T..C .CTA...CC. ...T...... ....A.C... AT....C.TT .TA..GC... .TTG.....C .G.A.G..CT ...T...... .....
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Fig. 1 (continued)

12 years (Cascorbi 2003; Gunn et al. 2008), which is concordant with ecological data (Hattour and Macías 2002; Mather et al. 1995). The estimated time since the population expansion began, calculated from $t=\tau/2u$, was roughly 0.1328 Mya for *T. orientalis* and 0.1868~0.2242 Mya for *T. maccoyii*.

Discussion

Thunnus thynnus and *T. orientalis* should be classified into two separate species. Gibbs and Collette (1967) indicated that *T. orientalis* and *T. thynnus* should be considered subspecies based on the comparative

Table 1 Intraspecific and interspecific nucleotide differences (k) (above the diagonal) and nucleotide diversity (Pi) (below the diagonal) based on mitochondrial cytochrome *b* sequence data

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1. N02	***	2	5	5	11	5	8	42	37	37	40	39	41	43	37	37	38	37	36	37	37	37
2. N03	0.0018	***	5	5	11	5	8	44	39	39	42	41	43	45	39	40	40	39	38	39	40	39
3. N04	0.0044	0.0044	***	2	8	4	7	41	36	36	41	38	40	42	36	37	37	36	35	36	37	36
4. N08	0.0044	0.0044	0.0018	***	8	4	7	41	36	36	41	38	40	42	36	37	37	36	35	36	37	36
5. N14	0.0096	0.0096	0.0070	0.0070	***	6	9	43	38	38	43	40	42	44	42	43	43	42	41	42	43	41
6. N17	0.0044	0.0044	0.0035	0.0035	0.0088	***	9	47	42	42	43	40	42	44	38	39	39	38	37	38	39	38
7. N22	0.0070	0.0070	0.0061	0.0061	0.0114	0.0061	***	46	41	41	46	43	45	47	41	42	42	41	40	41	42	41
8. S02	0.0368	0.0386	0.0359	0.0359	0.0412	0.0377	0.0403	***	7	7	14	9	11	13	13	14	14	13	12	13	14	13
9. S08	0.0324	0.0342	0.0316	0.0316	0.0368	0.0333	0.0359	0.0061	***	2	9	4	6	8	9	10	10	9	8	9	10	9
10. S09	0.0324	0.0342	0.0316	0.0316	0.0368	0.0333	0.0359	0.0061	0.0018	***	9	4	6	8	9	10	10	9	8	9	10	9
11. S15	0.0351	0.0368	0.0359	0.0359	0.0377	0.0368	0.0403	0.0123	0.0079	0.0079	***	7	9	11	16	17	17	16	15	16	17	16
12. S16	0.0324	0.0359	0.0333	0.0333	0.0351	0.0351	0.0377	0.0079	0.0035	0.0035	0.0061	***	4	6	11	12	12	11	10	11	12	11
13. S18	0.0359	0.0377	0.0351	0.0351	0.0368	0.0368	0.0394	0.0096	0.0053	0.0053	0.0079	0.0035	***	4	9	10	10	9	8	9	10	9
14. S19	0.0377	0.0394	0.0368	0.0368	0.0368	0.0368	0.0412	0.0114	0.0070	0.0070	0.0096	0.0053	0.0035	***	11	12	12	11	10	11	12	11
15. AB097669.1	0.0324	0.0342	0.0316	0.0316	0.0368	0.0333	0.0359	0.0114	0.0079	0.0079	0.0140	0.0096	0.0079	0.0096	***	3	3	2	1	2	3	2
16. AY302574.2	0.0324	0.0351	0.0324	0.0324	0.0377	0.0342	0.0368	0.0123	0.0088	0.0088	0.0149	0.0105	0.0088	0.0105	0.0027	***	2	3	2	3	0	3
17. EF427612.1	0.0333	0.0351	0.0324	0.0324	0.0377	0.0342	0.0368	0.0123	0.0088	0.0088	0.0149	0.0105	0.0088	0.0105	0.0027	0.0018	***	3	2	3	2	3
18. EF439243.1	0.0324	0.0342	0.0316	0.0316	0.0368	0.0333	0.0359	0.0114	0.0079	0.0079	0.0140	0.0096	0.0079	0.0096	0.0018	0.0027	0.0027	***	1	2	3	2
19. EF439609.1	0.0316	0.0333	0.0307	0.0307	0.0359	0.0324	0.0351	0.0105	0.0070	0.0070	0.0131	0.0088	0.0070	0.0088	0.0009	0.0018	0.0018	0.0009	***	1	2	1
20. EU036522.1	0.0324	0.0342	0.0316	0.0316	0.0368	0.0333	0.0359	0.0114	0.0079	0.0079	0.0140	0.0070	0.0079	0.0096	0.0018	0.0027	0.0027	0.0018	0.0009	***	3	2
21. NC 004901	0.0324	0.0351	0.0324	0.0324	0.0377	0.0342	0.0368	0.0123	0.0088	0.0088	0.0149	0.0105	0.0088	0.0105	0.0027	0.0008	0.0027	0.0027	0.0018	0.0027	***	3
22. NC 014052	0.0324	0.0342	0.0316	0.0316	0.0359	0.0333	0.0359	0.0114	0.0079	0.0079	0.0140	0.0096	0.0079	0.0096	0.0018	0.0027	0.0018	0.0018	0.0009	0.0018	0.0027	***

N and S respectively indicate *Thunnus orientalis* and *T. maccoyii*.

The data within species are indicated by gray shading

Table 2 Intraspecific and interspecific nucleotide genetic distances (above the diagonal) and amino acid genetic distances (below the diagonal) based on mitochondrial cytochrome *b* sequence data

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1. N02	***	0.002	0.004	0.004	0.010	0.004	0.007	0.038	0.033	0.033	0.036	0.035	0.037	0.039	0.033	0.033	0.034	0.033	0.032	0.033	0.033	0.033
2. N03	0	***	0.004	0.004	0.010	0.004	0.007	0.040	0.035	0.035	0.038	0.037	0.039	0.041	0.035	0.036	0.036	0.035	0.034	0.035	0.036	0.035
3. N04	0.005	0.005	***	0.002	0.007	0.004	0.006	0.037	0.033	0.033	0.037	0.034	0.036	0.038	0.032	0.033	0.033	0.032	0.032	0.032	0.033	0.032
4. N08	0.003	0.003	0.003	***	0.007	0.004	0.006	0.037	0.033	0.033	0.037	0.034	0.036	0.038	0.032	0.033	0.033	0.032	0.032	0.032	0.033	0.032
5. N14	0.008	0.008	0.008	0.005	***	0.009	0.012	0.043	0.038	0.038	0.039	0.036	0.038	0.040	0.038	0.039	0.039	0.038	0.037	0.038	0.039	0.037
6. N17	0.005	0.005	0.005	0.003	0.008	***	0.006	0.039	0.034	0.034	0.038	0.036	0.038	0.040	0.034	0.035	0.035	0.034	0.033	0.034	0.035	0.034
7. N22	0.011	0.011	0.011	0.008	0.013	0.011	***	0.042	0.037	0.037	0.042	0.039	0.041	0.043	0.037	0.038	0.038	0.037	0.036	0.037	0.038	0.037
8. S02	0.016	0.016	0.016	0.013	0.019	0.016	0.021	***	0.006	0.006	0.012	0.008	0.010	0.012	0.011	0.012	0.012	0.012	0.011	0.012	0.012	0.012
9. S08	0.008	0.008	0.008	0.005	0.011	0.008	0.013	0.008	***	0.002	0.008	0.004	0.005	0.007	0.008	0.009	0.009	0.008	0.007	0.008	0.009	0.008
10. S09	0.011	0.011	0.011	0.008	0.013	0.011	0.016	0.011	0.003	***	0.008	0.004	0.005	0.007	0.008	0.009	0.009	0.008	0.007	0.008	0.009	0.008
11. S15	0.011	0.011	0.011	0.008	0.011	0.011	0.016	0.011	0.003	0.005	***	0.006	0.008	0.010	0.014	0.015	0.015	0.014	0.013	0.014	0.015	0.014
12. S16	0.011	0.011	0.011	0.008	0.011	0.011	0.016	0.011	0.003	0.005	0	***	0.004	0.005	0.010	0.011	0.011	0.010	0.009	0.010	0.011	0.010
13. S18	0.011	0.011	0.011	0.008	0.011	0.011	0.016	0.011	0.003	0.005	0	0	***	0.004	0.008	0.009	0.009	0.008	0.007	0.008	0.009	0.008
14. S19	0.013	0.013	0.013	0.011	0.013	0.013	0.019	0.013	0.005	0.008	0.003	0.003	0.003	***	0.010	0.011	0.011	0.010	0.009	0.010	0.011	0.010
15. AB097669.1	0.005	0.005	0.005	0.003	0.008	0.005	0.011	0.011	0.003	0.005	0.005	0.005	0.005	0.008	***	0.003	0.003	0.002	0.001	0.002	0.003	0.002
16. AY302574.2	0.005	0.005	0.005	0.003	0.008	0.005	0.011	0.011	0.003	0.005	0.005	0.005	0.005	0.008	0	***	0.002	0.003	0.002	0.003	0	0.003
17. EF427612.1	0.005	0.005	0.005	0.003	0.008	0.005	0.011	0.011	0.003	0.005	0.005	0.005	0.005	0.008	0	0	***	0.003	0.002	0.003	0.002	0.003
18. EF439243.1	0.005	0.005	0.005	0.003	0.008	0.005	0.011	0.011	0.003	0.005	0.005	0.005	0.005	0.008	0	0	0	***	0.001	0.002	0.003	0.002
19. EF439609.1	0.005	0.005	0.005	0.003	0.008	0.005	0.011	0.011	0.003	0.005	0.005	0.005	0.005	0.008	0	0	0	0	***	0.001	0.002	0.001
20. EU036522.1	0.005	0.005	0.005	0.003	0.008	0.005	0.011	0.011	0.003	0.005	0.005	0.005	0.005	0.008	0	0	0	0	0	***	0.003	0.002
21. NC 004901	0.005	0.005	0.005	0.003	0.008	0.005	0.011	0.011	0.003	0.005	0.005	0.005	0.005	0.008	0	0	0	0	0	0	***	0.003
22. NC 014052	0.005	0.005	0.005	0.003	0.008	0.005	0.011	0.011	0.003	0.005	0.005	0.005	0.005	0.008	0	0	0	0	0	0	0	***

N and S respectively indicate *Thunnus orientalis* and *T. maccoyii*. Intraspecific data are indicated by gray shading

anatomy. However, Collette (1999) proposed that Atlantic and Pacific bluefin tunas be regarded as full species based on genetic and morphological evidence. In this study, we found that *T. thynnus* could be distinguished from other *Thunnus* species based on the 132nd nucleotide of the Cyt *b* gene (G) which was diagnostic for *T. thynnus* (Fig. 1). The 831st nucleotide of the Cyt *b* gene differed between *T. thynnus* and

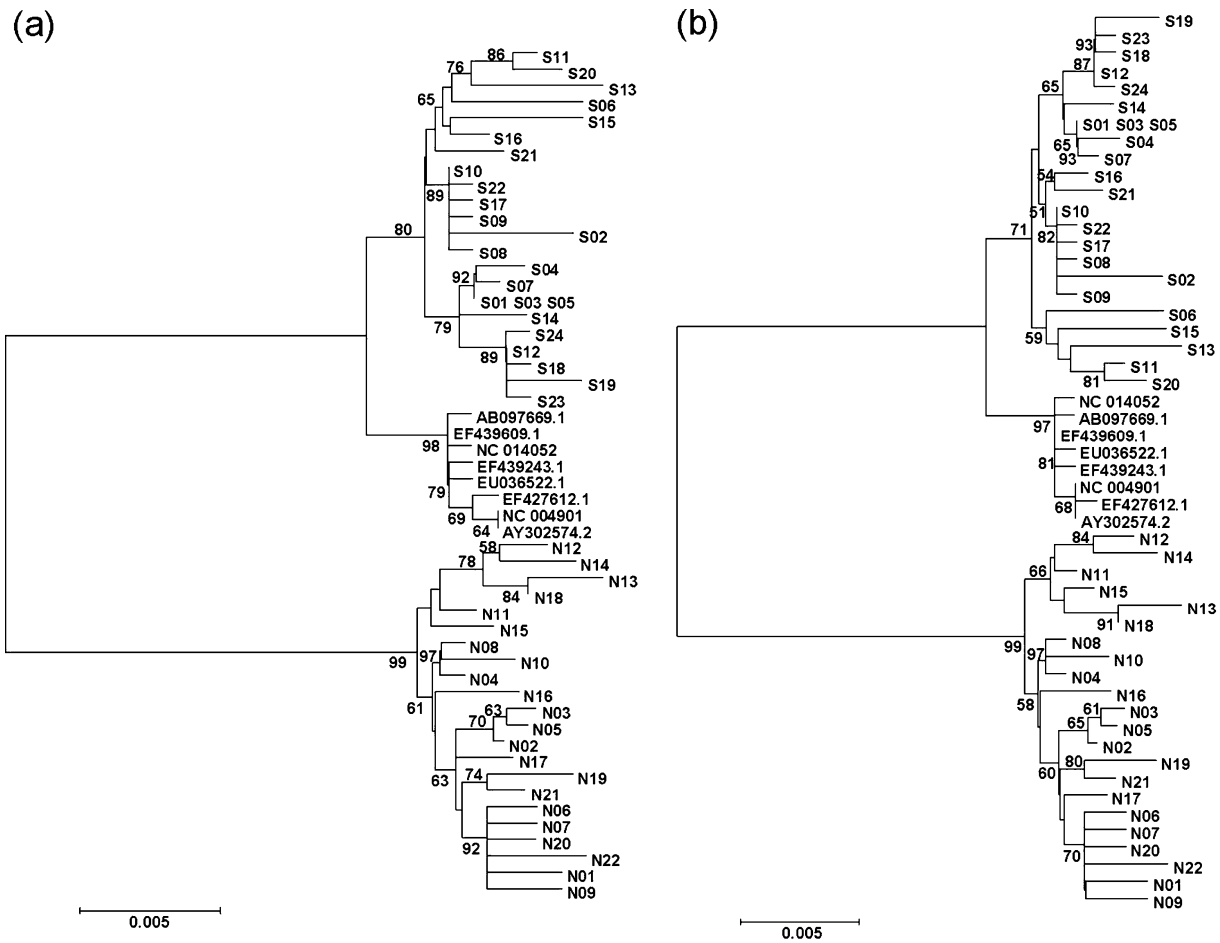

Fig. 2 **a** Neighbor-joining and **b** maximum-evolution phylogenetic trees constructed using the genetic distance method. Bootstrap values of >50% (out of 1,000 replicates) are shown at the nodes

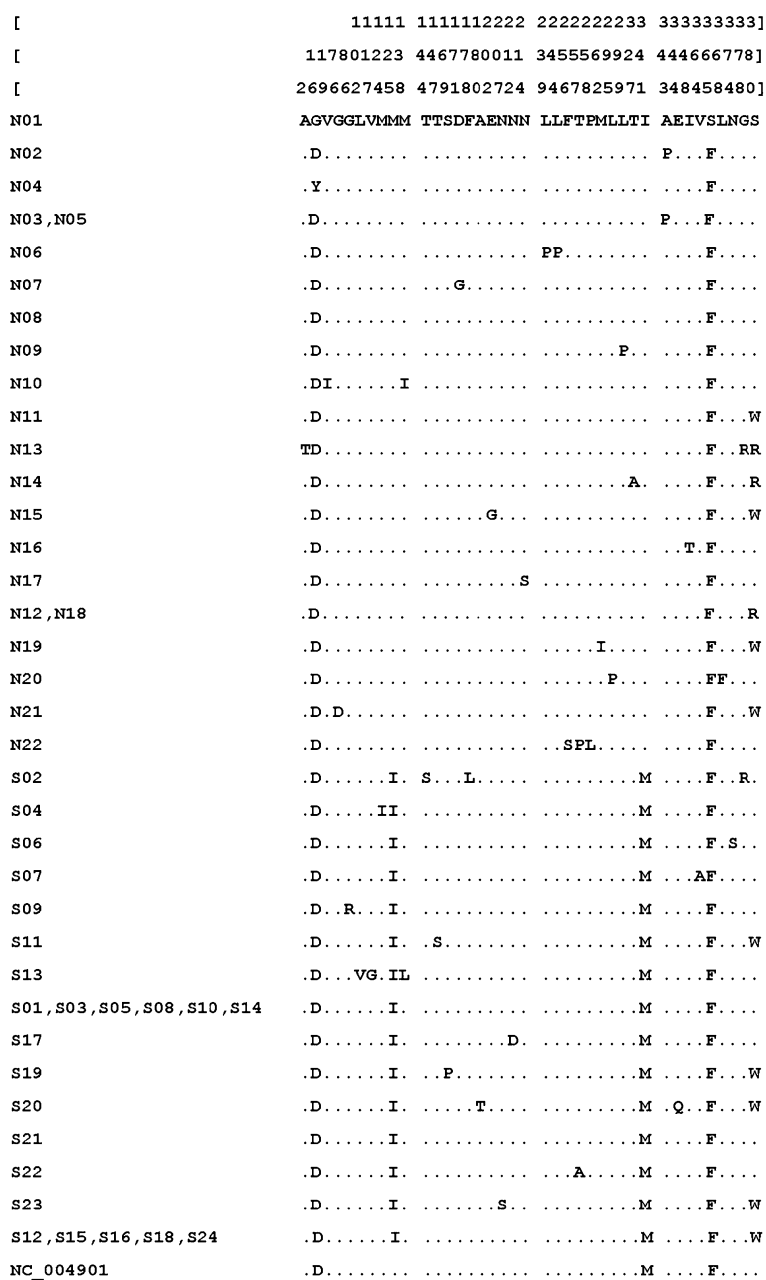

Fig. 3 Polymorphic sites in the 19 amino acid sequences of cytochrome *b* from *Thunnus orientalis* (N) and in 16 sequences from *T. maccoyii* (S). Samples N03 and N05 shared an identical sequence with N02. N12 and N18 shared an identical sequence. S01, S03, S05, S08, S10, and S14 had the same sequence. S12, S15, S16, S18, and S24 shared a common sequence. Eight nucleotide sequences from *T. thynnus* coded an identical amino acid sequence

T. orientalis (A vs. T). The NJ and ME trees also suggested that *T. thynnus* and *T. maccoyii* have a closer phylogenetic relationship than either with *T. orientalis*. The Cyt *b* gene is also an efficient genetic marker for distinguishing *T. orientalis* and *T. maccoyii* from other *Thunnus* species. For example, the 375th nucleotide (A) is diagnostic for *T. maccoyii*. The 1023rd nucleotide (A) is diagnostic for *T. orientalis* (Fig. 1). Both characters can be used to distinguish the respective species from other *Thunnus* species. Although these two species have overlapped distributions in the southern Pacific Ocean, results by Chow and Kishino (1995) based on mtDNA ATPase data revealed significant differentiation between the two species. We merely employed eight different Cyt *b* sequences of *T. thynnus* from NCBI's GenBank, but

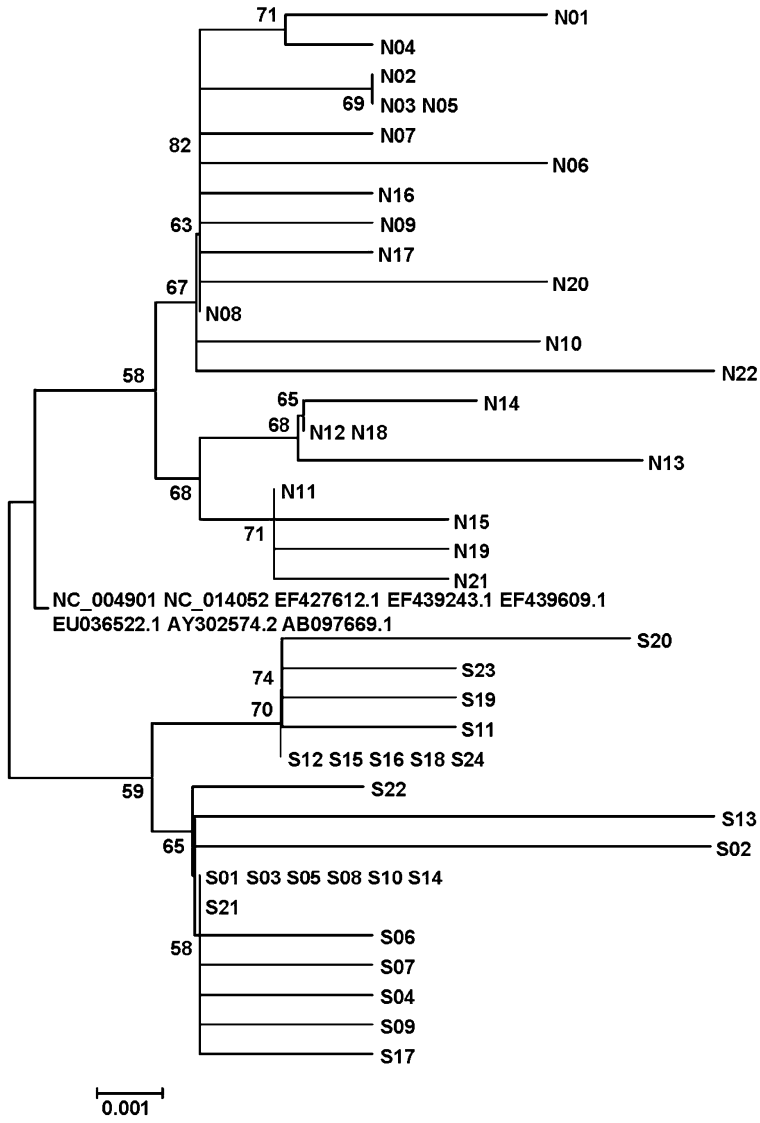

Fig. 4 The Neighbor-joining tree of cytochrome *b* amino acid sequences. Numbers above the branches indicate the bootstrap values

the results clearly showed that the Atlantic, Pacific, and Southern bluefin tunas should be defined as separate species.

Lengths of the complete Cyt *b* gene in tuna from this study ranged 1,138~1,141 bp and were similar to those of most fishes (Takehana et al. 2004; Zhao et al. 2008). Compared to the intraspecific nucleotide diversity of other fishes, that of a freshwater salangid was 0.0022 (Zhao et al. 2008), a tropical damselfish *Acanthochromis polyacanthus* was 0.045 (Planes et al. 2001), a coastal marine bonefish *Albula* sp. was 0.0036 (Pfeiler et al. 2008), and migratory *T. thynnus*, *T. orientalis,* and *T. maccoyii* were 0.0019, 0.0063, and 0.0059, respectively (this study). The migratory *T. orientalis* and *T. maccoyii* have higher nucleotide diversities than do freshwater and coastal fishes. They have a high H_d with moderate levels of sequence divergence between haplotypes, which suggests the accumulation of mutations in a rapidly growing population (Rogers and Harpending 1992). Unusually low nucleotide diversity exhibited within *T. thynnus* may have resulted from a small sample size or artificial overexploitation.

Assuming a sequence divergence of Cyt *b* in bony fishes of *ca.* 1.0%~1.8% per million years (Bermingham et al. 1997; Banford et al. 2004), we roughly estimated the times of species speciation to be 0.35~0.63 Mya for *T. orientalis* and 0.328~0.59 Mya

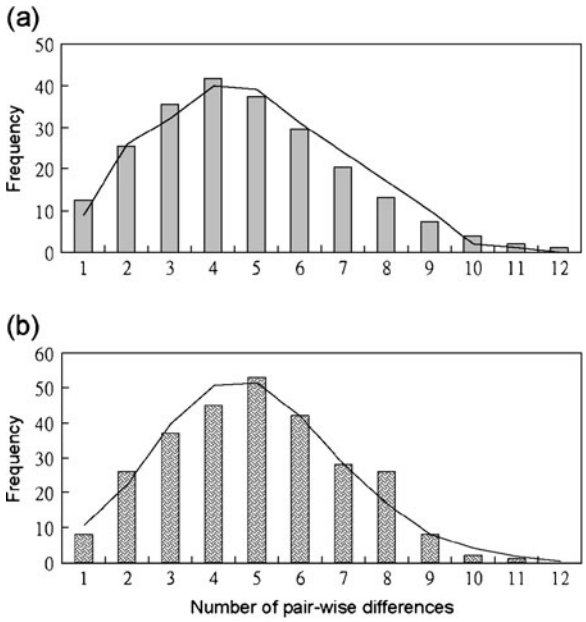

Fig. 5 Mismatch distributions obtained from mtDNA cytochrome *b* data. The bars of the histogram represent the observed pairwise differences. The curve is the expected distribution under a sudden expansion model. **a** Pacific bluefin tuna. **b** Southern bluefin tuna

for *T. maccoyii*. These inferences are similar to the results of Chow and Kishino (1995) who suggested that *T. maccoyii* diverged about 0.5 Mya.

Thunnus orientalis and *T. maccoyii* have undergone historical expansion in the North Pacific and Indian Oceans based on mismatch, Tajima's *D*, and Fu's F_s analyses, and tests of significance were used to assess the historical demographics (Fig. 5). Furthermore, the expansion times of *T. orientalis* and *T. maccoyii* were estimated to have been during the middle Pleistocene (0.126~0.781 Mya). There is increasing evidence that Pleistocene environmental changes, including sea level changes associated with glacial cycles (Haq et al. 1987), may have led to population expansions of marine organisms in the North Pacific and Indian Oceans. The MSTs from the Cyt *b* gene of both species also present net-shaped trees and most haplotypes being located at the tips of the MSTs suggests adaptative radiations of the populations (Fig. 6). In addition to *T. orientalis* and *T. maccoyii*, the mismatch distribution, Tajima's *D*, and Fu's F_s analyses also provided evidence of population expansion of the Cortez bonefish *Albula* sp. in the Gulf of California dating to the Pleistocene (Pfeiler et al. 2008).

The nucleotide diversity between *T. maccoyii* and *T. thynnus* (reference sequences) ranged 0.0061~0.0149 which overlaps with the intraspecific nucleotide diversity of *T. maccoyii* (0.0009~0.0123), suggesting a closer evolutionary relationship between these two species (Fig. 2). Nevertheless, a reverse result occurred in the analysis of amino acid sequences which showed a higher amino acid similarity between *T. thynnus* and *T. orientalis* (Fig. 4). The

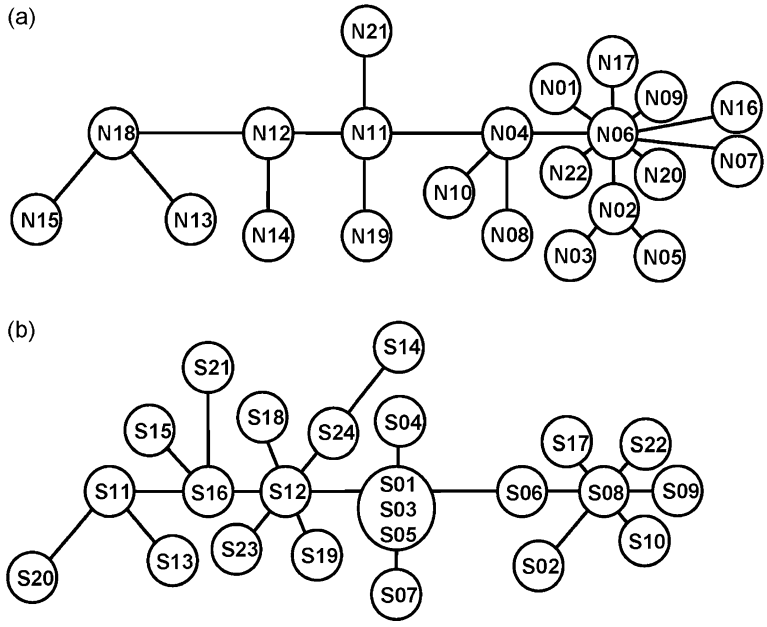

Fig. 6 Minimum spanning network of **a** 22 haplotypes of *Thunnus orientalis* and **b** 22 haplotypes of *T. maccoyii* reconstructed by nucleotide sequences of the cytochrome *b* gene. The size of the circles indicates the number of repetitive units belonging to the same haplotype. The length of the branches indicates the numbers of substitutions

present results of amino sequences are consistent with allozyme analyses by Sharp and Pirages (1978) and Elliott and Ward (1995), indicating a closer affinity of *T. orientalis* to *T. thynnus*. However, phylogenetic relationships based on nucleotide and amino acid sequences of these three bluefin tunas remain controversial. Chow et al. (2006) examined nucleotide sequence variations of ribosomal (r)DNA ITS1 and mtDNA data from all *Thunnus* species, and proposed mitochondrial introgression between species. From our results, analyses of nucleotide and amino acid sequences exhibited different phylogenetic relationships among the three bluefin tunas, and reflect how the expressions of genes are affected by the environment. Silent mutations are the major variation of the Cyt *b* gene between *T. orientalis* and *T. thynnus*, which maintains similar protein structures and functions.

Natural hybridization is believed to be more common in fish than in any other group of vertebrates (Jansson et al. 2006), and a number of gene introgressions were reported in fish (Smith 1992). Nuclear (n)DNAs of *T. orientalis* and *T. thynnus* are almost identical, but the mtDNAs greatly differ (Chow and Kishino 1995). Our results also present very dissimilar nucleotide compositions of the Cyt *b* gene between *T. orientalis* and *T. thynnus*. A high similarity of mtDNA between *T. orientalis* and *T. alalunga* suggests that interspecific hybridization occurred between those *Thunnus* species (Chow and Kishino 1995). Ranges of inter- and intraspecific discrepancies of mtDNA overlapped between *T. maccoyii* and *T. thynnus* in this study, but a significant difference occurred in an analysis of rDNA (Chow et al. 2006). These results indicate that *T. thynnus* and *T. maccoyii* shared a close common female ancestor, but *T. thynnus* and *T. orientalis* shared a close common male ancestor. This implies that *T. thynnus* may be a hybrid species which was derived after the appearance of *T. orientalis* and *T. maccoyii*. Chow et al. (2006) explained that male-biased gene flow is quite unlikely because very few *T. orientalis* individuals have been caught in the southern hemisphere. When we explored the past demographic history, we discovered population expansion of *T. orientalis* (since 0.1328 Mya) and *T. maccoyii* (since 0.1868~0.2242 Mya) during the middle Pleistocene. Distributions of marine species during interglacial and glacial periods depended on the availability of suitable current systems and the ecological characteristics of the species. However, we do not have accurate knowledge of the distribution of either species during that period. We cannot judge whether their distributions overlapped or not. From previous molecular evidence and results of this study, we inferred that historical interspecific hybridization may have taken place between these two species. More studies on tuna cytogenetics are required in order to clarify the evolutionary relationships of tuna species in the future.

Fish species identification has become an important concern, especially for high-value tuna species which are usually distributed as filleted, broiled, and canned products. Genetic markers play important roles in species identification when external morphological characteristics of fish are not available. In this study, we confirmed that the Cyt *b* gene is very useful for discriminating the cryptic species of *T. thynnus*, *T. orientalis,* and *T. maccoyii*. We also explored the historical demography of the latter two species and discovered recent population expansions dating to the middle Pleistocene.

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