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## Interspecific and Intraspecific Genetic Diversity of *Thunnus* Species

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### 1. Introduction

The genus *Thunnus* contains eight valid species with a fossil record extending back to the Middle Eocene, about 40 million years ago (Carroll, 1988, Benton, 1993). Collette (1978) distinguished tunas into the temperate subgenus *Thunnus* (bluefin tuna group) and the tropical subgenus *Neothunnus* (yellowfin group) by the presence or absence of a central heat exchanger. The yellowfin group contains blackfin (*T. atlanticus*), longtail (*T. tonggol*), and yellowfin (*T. albacares*) tunas. Albacore (*T. alalunga*), bigeye (*T. obesus*), northern bluefin (*T. thynnus* & *T. orientalis*), and southern bluefin (*T. maccoyii*) tunas are members of the bluefin group. *T. obesus* shares approximately the same number of characters with each group; however, it is classified as a member of the subgenus *Thunnus* since the character states consist of adaptations to life in colder environments (Collette, 1978).

Finnerty & Block (1995) explored *Thunnus* systematics using a portion of the cytochrome (*Cyt*) *b* gene. However, only five of eight tuna species were analyzed, and results were insufficient to draw conclusions about relationships within the genus *Thunnus*. Alvarado-Bremer et al. (1997) constructed phylogenetic relationships among tunas from a portion of the mitochondrial (mt) DNA control region. A Neighbor-joining tree supported monophyletic origins for the temperate subgenus *Thunnus* and of the tropical subgenus *Neothunnus*, except for bigeye tuna because it was difficult to place in either subgenus. This result is consistent with allozyme data suggesting that the bigeye tuna has a greater similarity to yellowfin and blackfin tunas than to temperate tunas (Sharp & Pirages, 1978; Elliott & Ward, 1995). Alvarado-Bremer et al. (1997), Ward (1995), and Chow & Kishino (1995) suggested that mtDNAs of albacore and Pacific bluefin tunas share a common ancestry. Nevertheless, Sharp & Pirages (1978) and Chow & Kishino (1995) indicated that albacore tunas are highly divergent from all other tunas, suggesting that the *Thunnus* subgenus is not a monophyletic group. Elliott and Ward (1995) reported that bluefin tunas were much closer to the yellow tuna than to albacore and bigeye tunas, and albacore was the most divergent species in the

genus. Alvarado-Bremer et al. (1997) inferred that introgression of albacore mtDNA into the Pacific bluefin tuna occurred a long time ago.

Chow et al. (2006) examined intra- and interspecific nucleotide sequence variations of the first internal transcribed spacer (*ITS1*) of ribosomal (r)DNA among all *Thunnus* species. Their report supported mitochondrial introgression between species and contradicted the morphological subdivision of the genus into the two subgenera, *Neothunnus* and *Thunnus*. The cladogram constructed from the *ITS1* indistinctly resolved phylogenetic relationships among three tropical tunas (*T. albacare*, *T. tonggol*, and *T. atlanticus*). The *ITS1* and mtDNA ATCO sequence data both supported the monophyletic status of the yellowfin tuna group and indicated that these tropical tunas are recently derived taxa; nevertheless, *T. thynnus* and *T. orientalis* shared almost identical *ITS1* sequences, while having distinct mtDNA. These molecular data suggested that intermittent speciation events occurred in species of the genus *Thunnus*. Consequently, relationships among closely related *Thunnus* taxa remain unresolved.

The Pacific bluefin tuna *T. orientalis* is a migratory pelagic fish of high commercial value in world fisheries (Ward et al., 1995). This species is primarily distributed in the North Pacific Ocean, from the Gulf of Alaska to southern California and Baja California and from Sakhalin Island in the southern Sea of Okhotsk to the northern Philippines; the tuna is also found in the South Pacific Ocean around Australia, the Galapagos Islands, the Gulf of Papua, and New Zealand (Bayliff, 1994, Collette & Nauen, 1983, Collette & Smith, 1981, Smith et al., 2001; Ward et al., 1995). Pacific bluefin tuna spawn between Japan and the Philippines, in the Sea of Japan south of Honshu (Chen et al., 2006; Itoh, 2009, Tanaka et al., 2006, Tanaka et al., 2007); larvae are transported northwards towards Japan by the Kuroshio Current, and juveniles are found in waters near Japan. Some young fish migrate east as far as the western coast of North America, and are presumed to return to the western Pacific to breed (Bayliff, 1991, 2001). The Pacific bluefin tuna is believed to become sexually mature at about 5 years of age and to have a maximum lifespan of 25 years (Bayliff, 1994; Ueyanagi, 1975). Surprisingly little is known about the stock structure and population biology of *T. orientalis*. Currently there are no data on the population genetics of *T. orientalis* stocks exploited in the northern and southern Pacific Ocean.

In the present study, we collected complete *Cyt b* sequences from all eight *Thunnus* species, described interspecies genetic variations of the *Cyt b* gene, and explored their phylogeny. Results indicated that *Cyt b* is a good tool for tuna identification. The *Thunnus* phylogeny did not conform to the two-subgenera classification pattern. Another aim of this research was to investigate whether genetic differentiation appears between *T. orientalis* samples from Taiwan and New Zealand waters. Results suggested that these two samples shared high genetic homogeneity.

## 2. Materials and methods

### 2.1 Tuna sampling

#### 2.1.1 Eight *Thunnus* tuna collection

All five *Thunnus* species *T. obesus*, *T. tonggol*, *T. alalunga*, *T. albacares*, and *T. orientalis* and an outgroup specimen *Katsuwonus pelamis* were collected from Taiwan waters. *Thunnus*

*maccoyii* was obtained from the Indian Ocean by Taiwanese fishery observers in 2006 as described by Shiao et al. (2008). *Thunnus thynnus* was caught from Martinique waters of the eastern Caribbean Sea in 2008. Tissue specimens of *T. atlanticus* were supplied by the Florida Museum of Natural History, Gainesville, FL, USA.

### 2.1.2 *Thunnus orientalis* sampling

Two sets of muscle tissue samples of *T. orientalis* were collected from the North and South Pacific Oceans. Forty specimens (T1~T40) were sampled from southeastern Taiwan waters (T) during 2006~2007 and 40 specimens (Z1~Z40) from New Zealand waters (Z) in 2001 by scientific observers on commercial fishing vessels (Fig. 1). Muscle tissue samples were preserved in 95% ethanol for DNA extraction.

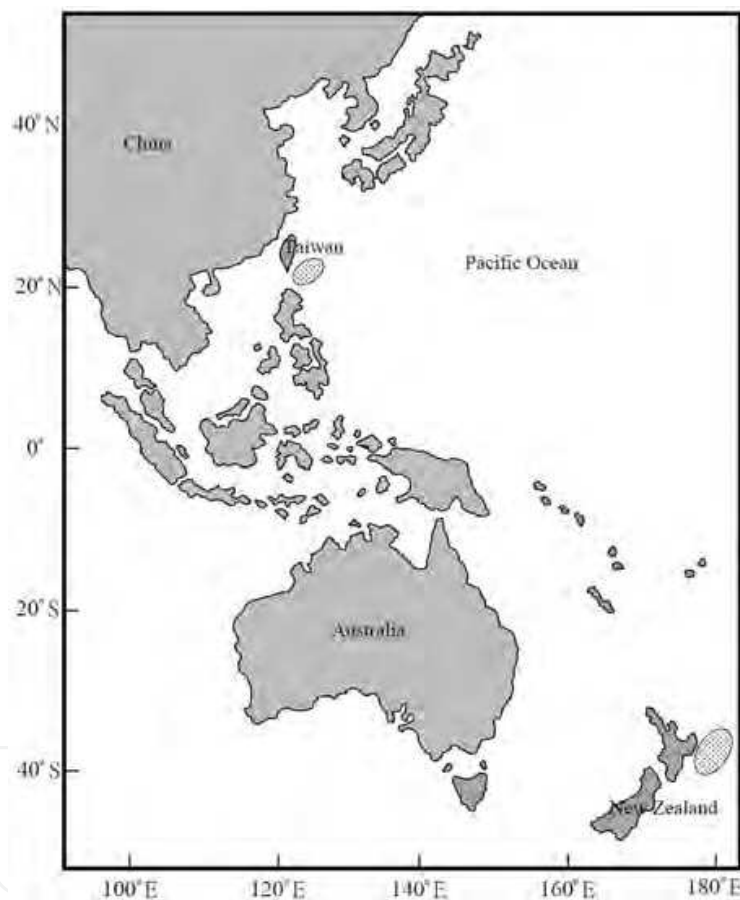


Fig. 1. Sampling locations of *Thunnus orientalis* in Taiwan and New Zealand waters.

## 2.2 DNA Cloning

### 2.2.1 DNA isolation and polymerase chain reaction (PCR) amplification

DNA was extracted as previously described (Kocher et al., 1989). Two sets of primers and amplification conditions were used to amplify the complete *Cyt b* gene and control region of mtDNA (Tseng et al., 2009, 2011). Amplification was performed in a BIO-RAD MJ Mini Gradient Thermal Cycler (Conmall Biotechnology, Singapore). Subsamples (at 10  $\mu$ L) of amplified products were checked on 0.8% agarose gels to confirm the product sizes.

## 2.2.2 DNA purification, transformation, and sequencing

The remaining successful PCR products were purified from agarose gels using a DNA Clean/Extraction kit (GeneMark, Taichung, Taiwan). Isolated DNA fragments were 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). Twenty individuals from each of the Taiwanese and New Zealand samples of *T. orientalis* were randomly chosen for cloning followed by sequencing in an Applied Biosystems (ABI, Foster City, CA, USA) automated DNA sequencer 377 (vers. 3.3) with a Bigdye sequencing kit (Perkin-Elmer, Wellesley, MA, USA). The T7 and SP6 primers were used in the sequencing reaction, and PCR cycle parameters for sequencing were 35 cycles of 95°C for 30s, 50°C for 30s, and 72°C for 1 min.

## 2.3 Sequence analyses

### 2.3.1 Cyt b gene analysis

Interspecific genetic diversities of nucleotides were calculated using MEGA software (Tamura et al., 2007). GTR+I+G was the best-fitting model for DNA substitutions as determined by the Modeltest 3.7 program (Posada & Crandall, 1998) using the Akaike information criterion (AIC = 5091.39, gamma = 0.5623). The phylogenetic tree of nucleotide sequences was constructed using the Neighbor-joining (NJ) method (Nei & Kumar, 2000). The confidence of the clusters was assessed using a bootstrap analysis with 1000 replications. Genetic distances between amino acid sequences were estimated using Poisson correction methods (Nei & Kumar, 2000) in MEGA (Tamura et al., 2007). The phylogenetic tree of amino acid sequences was constructed by the NJ method with an anterior-branch test, and the confidence of the clusters was assessed by 1000 bootstrap replications.

### 2.3.2 Control region analysis

In total, 40 nucleotide sequences of the mtDNA control region from the two sets of samples (T and Z) were aligned with the program, CLUSTAL W (Thompson et al., 1994), and verified by eye. All 40 sequences were deposited in NCBI GenBank (with accession nos. JN631171~1190 and JN631211~1230). The number of polymorphic sites including substitutions and indels was estimated using Arlequin vers. 3.1 (Excoffier et al., 2010). Levels of inter- and intra-sample genetic diversity were quantified by indices of  $h_d$  and pairwise estimates of nucleotide divergence ( $d_{ij}$ ), both among and within samples. The average number of nucleotide substitutions per site ( $\pi$ ) and pairwise differences between samples were determined using the DnaSP program (Librado & Rozas, 2009). Intraspecific genetic distances were analyzed by Kimura's 2-parameter (K2P) model (Kimura, 1980). The best-fitting models of substitution were determined using MODELTEST 3.7 (Posada & Crandall, 1998) with the AIC. The TVM+I+G model with gamma = 0.724 and AIC = 5370.36 was chosen as the best-fitting model. Phylogenetic trees were constructed using the NJ method (Nei & Kumar, 2000), and the confidence of each node of the tree was tested by bootstrapping (Felsenstein, 1985) with 1000 replicates. Nodes with bootstrap values of >70 were significantly supported by a  $\geq 95\%$  probability (Hillis & Bull, 1993). A minimum spanning tree (MST) was computed from the matrix of pairwise distances between all pairs of 40 haplotypes using a modification of the algorithm described by Rohlf (1973). An exact test of genetic



differentiation between samples was also estimated with  $F_{ST}$  (Raymond & Rousset, 1995) with  $10^5$  steps of a Markov chain.

## 2.4 Genotyping of microsatellites

Genetic variation in *T. orientalis* samples was estimated with six microsatellite loci: Tth14, -31, -185, -217, -226, and Ttho-4. The primers for these loci were developed by Clark et al. (2004) and Takagi et al. (1999). PCR cocktails consisted of ~1 ng genomic DNA, 10 pmol reverse primer, 10 pmol labeled forward primer, 25 mM dNTP, 0.05~0.1 mM  $MgCl_2$ , 10× buffer, and 0.5 U *Taq* polymerase (Takara Shuzo, Tokyo, Japan) made up to a 25- $\mu$ L volume with Milli-Q water. Forward primers were labeled with the FAM, TAMRA, or HEX fluorescence markers. Polymerase chain reaction (PCR) amplifications were carried out in a BIO-RAD MJ Mini Gradient Thermal Cycler (Conmell Biotechnology, Singapore) programmed with the following schedule: 1 cycle of 95°C for 4 min, followed by 35 cycles of 94°C for 30s, annealing at 52~60°C for 30s, and 72°C for 30s. Each 5  $\mu$ L of PCR production from three loci labeled with different fluorescence tags, was mixed and precipitated with 95% alcohol. Semiautomated genotyping was performed using a capillary MegaBACE-500 DNA analysis system (Amersham Biosciences, Piscataway, NJ, USA). Genotypes were scored with Genetic Profiler 1.5 (Amersham Biosciences). The overall genotype success rate was 100%. The size of each allele was checked by eye, and the Micro-checker software package (Van Oosterhout et al., 2004) was used to correct genotyping errors, such as non-amplified alleles, short allele dominance, and scoring of stutter peaks.

## 2.5 Microsatellite genotypic analyses

The total number of alleles ( $n_a$ ), allelic frequencies, and Shannon index were estimated for each locus using the program POPGENE (Yang & Yeh, 1993). Observed ( $H_O$ ) and expected ( $H_E$ ) heterozygosities were calculated for each locus (Raymond & Rousset, 1995). Deviations from Hardy-Weinberg equilibrium (HWE) were examined by an exact test using GENEPOP (Raymond & Rousset, 1995). Genetic differentiation between samples was characterized using  $F_{ST}$  and  $R_{ST}$  values as implemented in ARLEQUIN 3.1 (Schneider et al., 2000). A factorial correspondence analysis was performed with GENETIX (Belkhir et al., 1996-2004) to plot multilocus genotypes of the two samples in two dimensions.

## 3. Results

### 3.1 Genetic variations and genealogy of the *Cyt b* gene

The mitochondrial *Cyt b* gene was completely sequenced for all eight tuna individuals examined. Of the 1141 aligned base pairs, 20 sites were singleton variable and 45 sites were parsimoniously informative. The *Cyt b* gene cloned from *T. tonggol* and homological sequence EF141181.1 shared an identical sequence. Some individual nucleotide compositions only appeared in specific species except *T. thynnus* (Fig. 2). For example *T. tonggol* could be individually distinguished from each other *Thunnus* tuna by the 45<sup>th</sup>, 81<sup>st</sup>, 321<sup>st</sup>, 360<sup>th</sup>, and 762<sup>nd</sup> sites of the *Cyt b* sequences. *T. albacares* could be particularly distinguished from each other *Thunnus* tuna by the 330<sup>th</sup> and 783<sup>rd</sup> sites.

```

[
[
1112222 3333333333 4444444555 5555666677 7777777777 7888888999 999000000]
[
4680360237 0223556678 0144679012 2788012701 1345567788 9134459123 679022235]
[
5312251249 3140470357 5747878495 8328281851 7810624739 2916987216 653803950]
T. thynnus CTCCACCCC ACAACACGT ATCTCTATCC CTTCACTCGA CCTATCTCAT TTAGCCTTGG ACCCTGAGC
NC_004901.2 ...TG...TT .....G... ..C..... .....T..... .....
T. tonggol T.T..... .T...T... G..... .....T.... ..C.CTC.C. CC..... .....G..
EF141181.1 T.T..... .T...T... G..... .....T.... ..C.CTC.C. CC..... .....G..
T. maccoyii .....TT .....A. ....C.C.... .....T.... ..... ..GA..... G...C....
NC_014101.1 .....TT .....A. ....C.C.... .....T.... ..... ..G..... G...C....
T. alalunga .C...T... C.G...T... .C.CTCG.TT .C...T..AG ..GC...C CCTAT.CCA. ...A..GAT
NC_005317.1 .C...T... C.G...T... .C.CTCG..T .C.T.T...G ..GC...C CCTAT.CCA. ...A..GAT
T. orientalis .C...T... C.G.G..T... .CTCTCG.TT .CC..T...G T..GC..T.C CCTAT.CCA. ...A..AGAT
GU256524 .C...T... C.G.G..T... .CTCTCG.TT .C...T...G T..GC...C CCTAT.CCA. ...A..AGAT
T. albacares .....G..... .....T..C... ..... ..C.C...T. C..... ..G..
NC_014061.1 .....G..... .....T..... ..... ..C.C...T. C..... ..G..
T. obesus .....T.T .....A...C .....C.... ..TGTCT.. .TC.C...C. C...T.... ..G..
NC_014059.1 .....T .....A.T... .....G... ..T.T.... .TC.C...C. C...T...A .TA...G..
T. atlanticus .....T... .....G...T.... T..... ..C.C.C.C. C..... ..G..

```

Fig. 2. Total of 69 variable sites within 15 *Cyt b* sequences from eight tunas.

Numbers of different nucleotide between *Thunnus* species ranged from five (*T. alalunga* vs. GU256524) to 43 (*T. obesus* vs. *T. orientalis*), and interspecific K2P distances ranged 0.004~0.039 (Table 1). *Katsuwonus pelamis* had significant different nucleotide components from *Thunnus* tunas. The different nucleotide numbers between *K. pelamis* and *Thunnus* species ranged 122~128, and K2P distances ranged 0.117~0.123. Complete *Cyt b* sequences from all tuna species except *T. atlanticus* (wanting) were taken from NCBI (NC\_014101.1, NC\_004901.2, NC\_005317.1, NC\_014061.1, NC\_014059.1, EF141181.1, and GU256524) and selected as reference sequences in the phylogenetic analysis. These reference sequences and eight congeneric sequences from the study were consistently clustered in the NJ tree. The tree contained two explicit clades, the first group consisted of *T. maccoyii*, *T. thynnus*, *T. atlanticus*, *T. albacares*, *T. obesus*, *T. tonggol*, and the second group included *T. alalunga* and *T. orientalis*, with extremely high bootstrapping (1000 replications) support (Fig. 3). All eight tuna individuals and seven reference sequences coded five different amino acid sequences with high conservation. The *Cyt b* gene sequence of our *T. maccoyii* specimen and its reference sequence as well as *T. orientalis* and its reference sequence coded identical amino acid sequences. One identical amino acid sequence was shared by nine specimens from five *Thunnus* species, *T. alalunga*, *T. thynnus*, *T. albacares*, *T. atlanticus*, and *T. tonggol*. Poisson correction distances between different amino acid sequences ranged 0.003~0.005. Different amino acid numbers between sequences ranged 1~2. All branches presented an insignificant cluster from eight *Thunnus* species with slightly high bootstrapping support suggesting that parallel evolution had occurred in the protein structure of the *Cyt b* gene.

### 3.2 D-Loop genealogy

In total, 52 mutations were shared among the samples, and no fixed differences occurred in particular samples. Forty-one mutations were polymorphic in sample T, but monomorphic in sample Z. In contrast, 49 mutations were polymorphic in sample Z, but monomorphic in sample T. The average number of nucleotide differences between these two samples was 19.79. The average number of nucleotide substitutions per site between these two samples ( $D_{xy}$ ) was 0.023. Nucleotide ratios of the control region displayed a very high AT-rich composition (62%). A short double-repeat unit (TGCATGTGCAT) located at positions 47~57

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. <i>T. thunnus</i>	*	7	17	16	14	10	7	31	30	36	34	8	7	9	122
2. NC_004901.2	0.006	*	20	19	18	9	8	34	33	39	37	15	14	16	128
3. <i>T. tonggol</i>	0.015	0.018	*	11	19	21	22	38	35	43	41	16	15	16	126
4. <i>T. maccoyii</i>	0.014	0.017	0.01	*	18	22	21	35	32	40	38	15	14	15	127
5. NC_014101.1	0.012	0.016	0.017	0.016	*	22	21	35	34	40	38	13	12	11	122
6. <i>T. alalunga</i>	0.009	0.008	0.019	0.02	0.02	*	3	32	31	37	35	18	17	19	127
7. NC_005317.1	0.006	0.007	0.02	0.019	0.019	0.003	*	35	34	40	38	15	14	16	127
8. <i>T. orientalis</i>	0.028	0.031	0.034	0.032	0.032	0.029	0.032	*	3	7	5	31	30	32	122
9. GU256524	0.027	0.030	0.032	0.029	0.031	0.028	0.031	0.003	*	8	6	30	29	31	123
10. <i>T. albacares</i>	0.033	0.035	0.039	0.036	0.036	0.033	0.036	0.006	0.007	*	2	36	35	37	128
11. NC_014061.1	0.031	0.033	0.037	0.034	0.034	0.032	0.034	0.004	0.005	0.002	*	34	33	35	126
12. <i>T. obesus</i>	0.007	0.013	0.014	0.013	0.012	0.016	0.013	0.028	0.027	0.032	0.031	*	1	6	123
13. NC_014059.1	0.06	0.012	0.013	0.012	0.011	0.015	0.012	0.027	0.026	0.032	0.030	0.001	*	5	122
14. <i>T. atlanticus</i>	0.008	0.014	0.014	0.013	0.010	0.017	0.014	0.029	0.028	0.033	0.032	0.005	0.004	*	123
15. <i>K. pelamis</i>	0.117	0.123	0.121	0.122	0.117	0.122	0.122	0.117	0.118	0.123	0.121	0.118	0.117	0.118	*

Table 1. Numbers of different nucleotides between tuna species (upper diagonal) and K2P genetic distances of nucleotide sequences (below the diagonal) from *cytochrome b* gene sequences of eight *Thunnus* species and the outgroup *Katsuwonus pelamis*.

nt was observed in six individuals of *T. orientalis*. In total, 133 mutant sites containing 128 transitions, 14 transversions, and 14 indels were observed among the 40 haplotypes.

Long fragments, 865~876 base pairs (bp), containing the entire mtDNA control region were successfully amplified. Following alignment, the consensus nucleotide sequence for all specimens of *T. orientalis* was 894 bp long. Forty different sequences were found in the 40 individuals from T and Z samples with a very high haplotype diversity (100%). The number of different nucleotides ranged 2 (T5 vs. T6) to 31 (T1 vs. Z28) (Fig. 4). The nucleotide diversity ( $d_{ij}$ ) among sequences ranged 0.002 (T9 vs. Z19) to 0.040 (T1 vs. Z29) with an average of  $0.023 \pm 0.008$  (Table 2).

K2P genetic distances among different haplotypes ranged 0.002~0.042 with an overall average of  $0.023 \pm 0.003$ . The average number of nucleotide differences among all specimens was  $16.879 \pm 2.068$ . The  $d_{ij}$  between these two samples was  $0.023 \pm 0.002$ . Genetic distances ranged 0.002 (T5 vs. T6) to 0.035 (T1 vs. T19), with an average of 0.021 in sample T, and ranged 0.003 (Z19 vs. Z33) to 0.035 (Z13 vs. Z29) with an average of 0.02 in sample Z. The topology of the NJ tree was estimated using the entire dataset with a non-significant



geographical group (Fig. 5). Specimens from both sets of samples were scattered throughout the NJ tree. The pairwise  $F_{ST}$  value between these two samples was 0.009 ( $p = 0.244$ ). These results indicated that no specimens of *T. orientalis* exhibited genetic differentiation. The MST was computed from a matrix of pairwise distances between all pairs of haplotypes of *T. orientalis* (Fig. 6). Two central haplotypes were found in the MST (T15 and T11), and most of haplotypes were located at the tip of the MST.

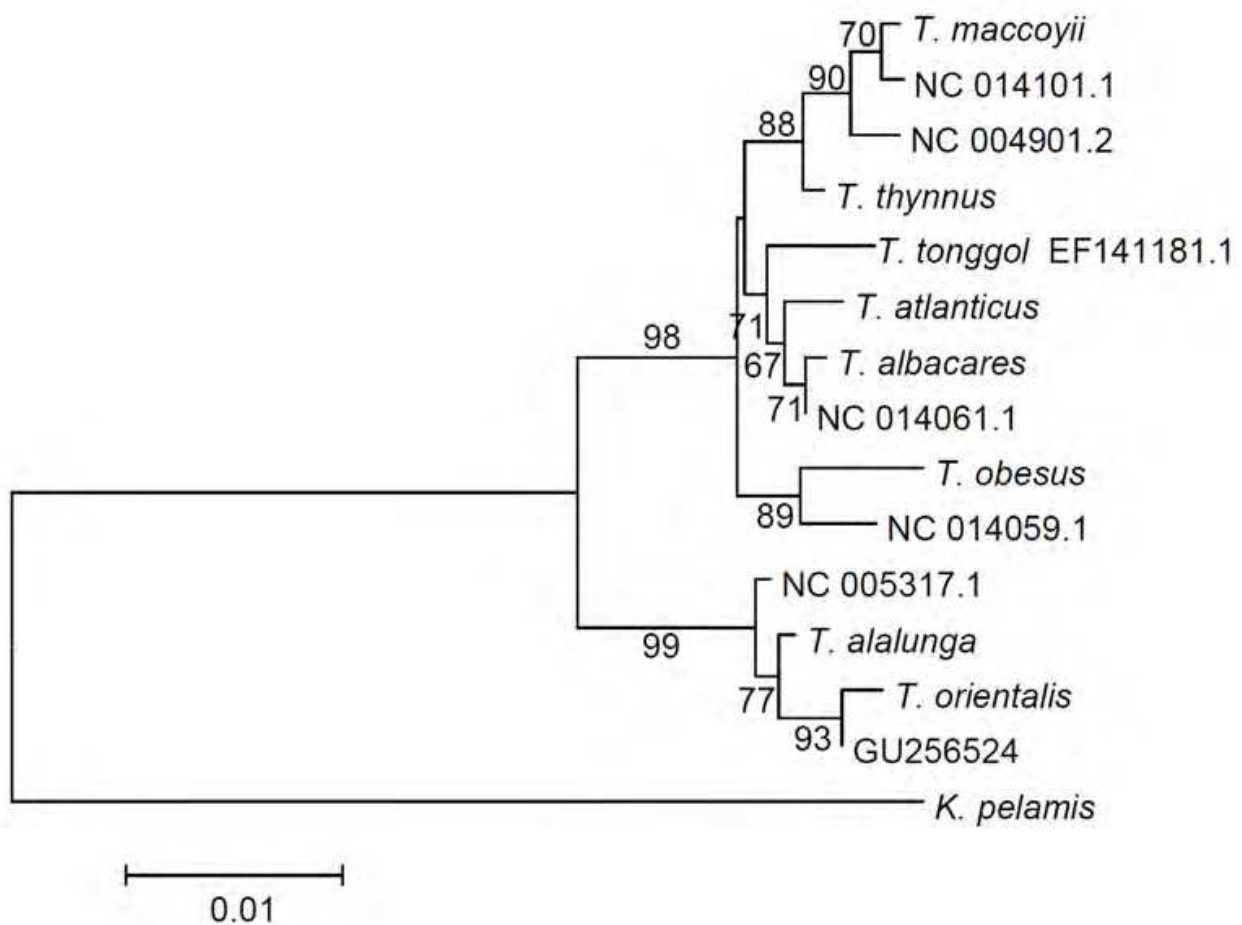


Fig. 3. Neighbor-joining tree constructed by 16 *Cyt b* gene sequences from eight *Thunnus* species and the outgroup *Katsuwonus pelamis*.

### 3.3 Microsatellite diversity

All six microsatellite loci were scored in these two sets of samples of *T. orientalis*, which had high levels of polymorphism (Fig. 7). The number of alleles per locus ranged 4 (Tth 185) to 19 (Tth 217) with an average of  $10.50 \pm 5.32$  ( $n = 80$ ). Shannon's information index ( $I$ ) estimated for different loci ranged 0.906~2.165 (mean,  $1.501 \pm 0.410$ ) in sample T, and 0.693~2.544 (mean,  $1.632 \pm 0.624$ ) in sample Z. Mean numbers of alleles ( $na$ ) per locus were  $7.833 \pm 3.833$  for sample T and  $9.833 \pm 5.565$  for sample Z (Table 3).

```

[          11111 11111111111 1111122222 2222222222 2222222222]
[ 468901234 4456666789 9999900000 1111122234 4445556667]
[ 9424496541 2682357671 3678901239 0124582613 5692360574]
T1      TGTTAATCAC CCCACTTAGA CTTATTCTTT TCTGTCAAAA TCCTACATCT
T5      ..... T.T..... ..... C..... .....
T9      .A....TG. T.TC..C..G .....CT... ..TC...G ..A..G...
T11     C.....T.. T.T...C..G ..... C.....G .....T.
T17     .AC....T.. T.T..CCG.G .....CT... C..T.T..G .....T.
T22     .....G.T.. T.T.....G T.C..C.... C...C...G .....CT.
T25     .A....T.. T.T...C..G T.C..CT.C. C.CCC...G .....T.
Z8      .A....T.. T.T...C..G T....CTC.. C..CC..G.G C.A.G..CT.
Z13     .....T.. T.T....G.. ..... C...C..... .....
Z24     .....T.. T.T...C..G .....CT... C..A....G .....T.
Z28     .A....T.. T.T.T.C... .....CT... C..CC...G C.....T.
Z34     .A....T.. T.T...C..G .....CT... C..C.T..G .....T.
Z36     .A....TG. T.TC..C..G .....CT... CT.TC...G .TAC...T.
Z39     .....T.. TTT.....G ..... C...C...G C.....TC
T. alalunga .A..CG.A.T T.T..... TCC....CCC .T.T.TG..G ..A..T..T.
T. thynnus  .A.ATGCA.T T.T.....A. .CCTC..CCC .T.T.T..GG C.A....T.

[          2223333333 3334444444 4555555555 5666666666 6777778888]
[ 8990001133 7780112345 8000223679 9014445667 906788356]
[ 6470141267 7813160957 3129195971 5431245123 060716648]
T1      TGACAAAGCT CGTCGTTGTT ACCGATATTA TGATATATAC TTCGTCTAT
T5      ..... ..... ..A....C. ....
T9      ...TG..... .A..... ..AG.GC.. .....G... ..T...
T11     ...TG..... .A..... ..A..GC.G ..... ..TC..
T17     C..TGG.... T.....A.C G..A...C.. ..... ..T...
T22     C...G..... .A...C.... ..A..GC.. ..... ..T...
T25     ...G..... T.....A.C ..A...C.. ...G..... ..T...
Z8      ..GT..... TA...C.A.. G..A..GC.. ..... ..T...
Z13     ...T..... ...T...C. ...A...C.. .....
Z24     ...TG..... ...C.... G..A...C.G ..... ..T..C
Z28     .AGT...A.. TA.T...A.. G..A.CGC.G ..C.C.... C...CT...
Z34     C..TG..... T.....A.C G..A...C.. ..... ..AA.T...
Z36     ...TG..... .A.A..... ...AG.GC.G ..... C...T...
Z39     ...T..... ..... G..A..... .AG.....T .....T.G.
T. alalunga .A.T..T.TC TA.T..CA.. .TTA..G.CG .....CG. .A...T...
T. thynnus  .A.T..T.TC TACT...A.. .TTA..G.C. C..... .A...T...

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Fig. 4. Variable sites within 16 selected D-loop sequences from 14 Pacific bluefin tuna specimens and two outgroups.

	T1	T5	T9	T11	T17	T22	T25	Z8	Z13	Z24	Z28	Z34	Z36	Z39	<i>T. alalunga</i>	<i>T. thynnus</i>
T1	*	5	24	18	27	21	25	31	11	20	33	25	30	30	41	43
T5	0.006	*	23	15	24	18	22	28	8	17	30	22	27	16	38	40
T9	0.028	0.027	*	16	21	19	20	20	21	17	26	20	10	25	42	44
T11	0.021	0.018	0.019	*	19	13	19	21	15	10	23	17	16	17	38	42
T17	0.032	0.029	0.025	0.022	*	22	15	21	22	14	25	7	23	24	43	45
T22	0.025	0.021	0.022	0.015	0.026	*	16	18	18	15	28	20	21	20	39	43
T25	0.030	0.026	0.024	0.022	0.018	0.019	*	18	22	16	24	12	22	24	40	44
Z8	0.037	0.033	0.024	0.025	0.025	0.021	0.021	*	26	18	18	18	22	24	40	42
Z13	0.013	0.009	0.025	0.018	0.026	0.021	0.026	0.031	*	17	26	22	25	16	41	43
Z24	0.024	0.020	0.020	0.012	0.016	0.018	0.019	0.021	0.020	*	22	12	17	17	43	47
Z28	0.040	0.036	0.031	0.027	0.030	0.033	0.029	0.021	0.031	0.026	*	22	24	28	42	44
Z34	0.030	0.026	0.024	0.020	0.008	0.024	0.014	0.021	0.026	0.014	0.026	*	22	22	42	44
Z36	0.036	0.032	0.012	0.019	0.027	0.025	0.026	0.026	0.030	0.020	0.028	0.026	*	27	42	46
Z39	0.022	0.019	0.030	0.020	0.029	0.024	0.029	0.028	0.019	0.020	0.033	0.026	0.032	*	45	45
<i>T. alalunga</i>	0.050	0.046	0.051	0.046	0.053	0.047	0.049	0.049	0.050	0.052	0.051	0.051	0.051	0.055	*	17
<i>T. thynnus</i>	0.052	0.048	0.053	0.051	0.055	0.052	0.053	0.051	0.052	0.057	0.053	0.053	0.056	0.055	0.020	*

Table 2. Numbers of different nucleotides of the mtDNA D-loop region (above the diagonal) and K2P genetic distances of nucleotide sequences (below the diagonal) among tuna specimens.

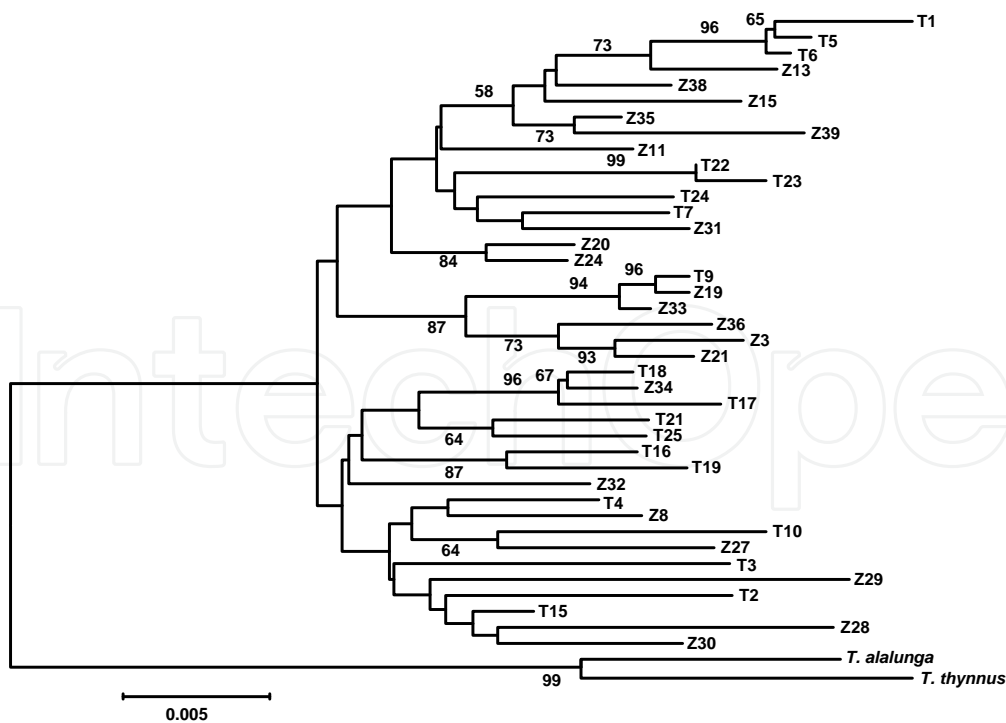


Fig. 5. Neighbor-joining tree constructed with 40 D-loop sequences from 20 individuals each from Taiwan and New Zealand waters. *Thunnus alalunga* and *T. thynnus* were selected as outgroups in the tree.

Four private alleles were found in sample T and 16 private alleles in sample Z. At the Tth-217 locus, there was a greater number of private alleles in sample T than in the other loci. The value of  $H_O$  over six loci ranged 0.425 (Tth-31) to 0.725 (Tth-217), with an average of  $0.633 \pm 0.127$  in sample T and ranged 0.2 (Tth-185) to 0.75 (Ttho-4, Tth-27, and Tth-226), with an average of  $0.613 \pm 0.217$  in sample Z. Values of  $H_E$  over six loci ranged 0.531 (Tth-185) to 0.872 (Tth-217), with an average of  $0.712 \pm 0.109$  in sample T and ranged 0.461 (Tth-185) to 0.911 (Tth-217), with an average of  $0.730 \pm 0.154$  in sample Z. Overall mean  $H_O$  and  $H_E$  values for the six loci were  $0.623 \pm 0.167$  and  $0.722 \pm 0.130$  (Table 3).

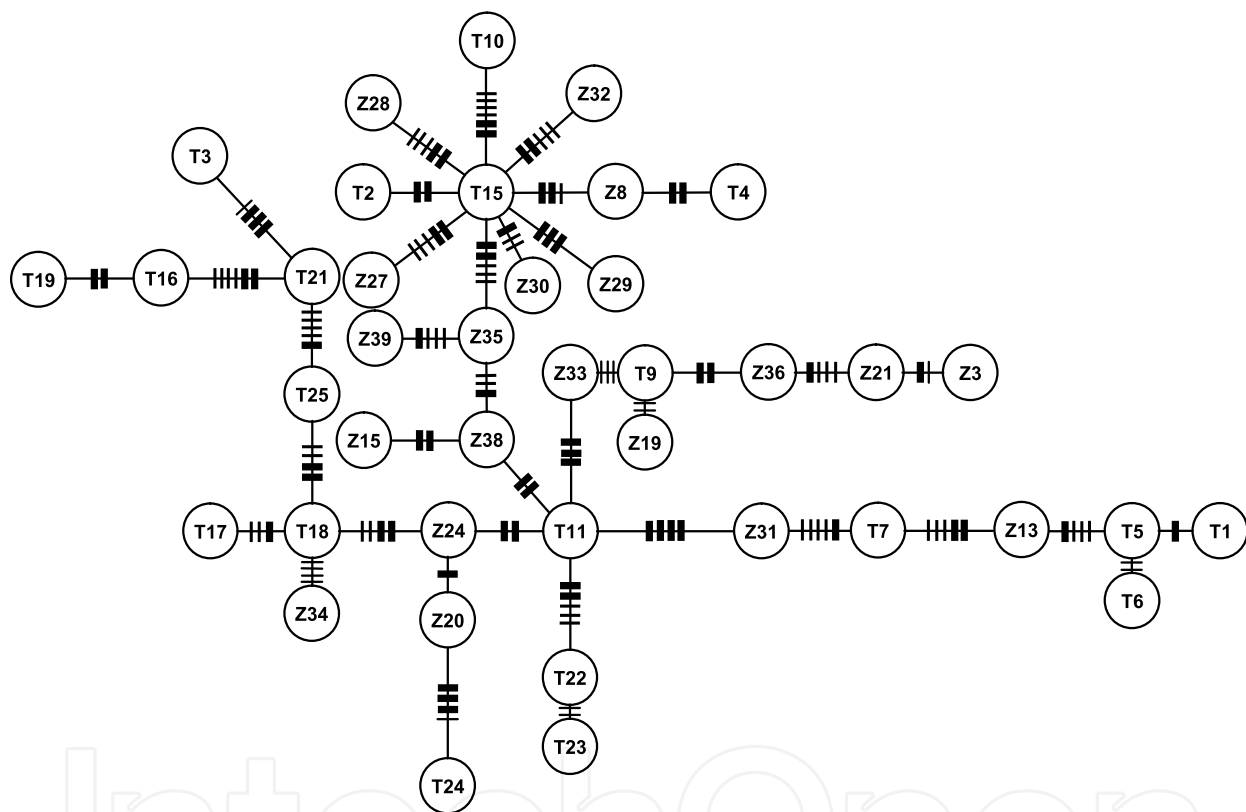


Fig. 6. Minimum spanning tree constructed from D-loop data.

Nei's genetic identity and genetic distance between the two samples were 0.978 and 0.222. Shannon's information index ranged 0.818 (Tth-185) to 2.435 (Tth-217) with an average of  $1.609 \pm 0.528$ . Permutation tests for linkage disequilibrium among the six loci for the two samples revealed only very slight disequilibrium for the entire dataset ( $D_{IT}^2 = 0.009$ ). The genetic differentiation index,  $F_{ST}$ , ranged 0.001 (locus Tth-14) to 0.016 (locus Tth-31) within loci and averaged 0.076.  $F_{ST}$  and  $R_{ST}$  values between these two samples were 0.003 ( $p = 0.243$ ) and 0.019 ( $p = 0.099$ ), respectively. A factorial correspondence analysis showed that these two samples had distributions that almost completely overlapped to a great extent (Fig. 8).

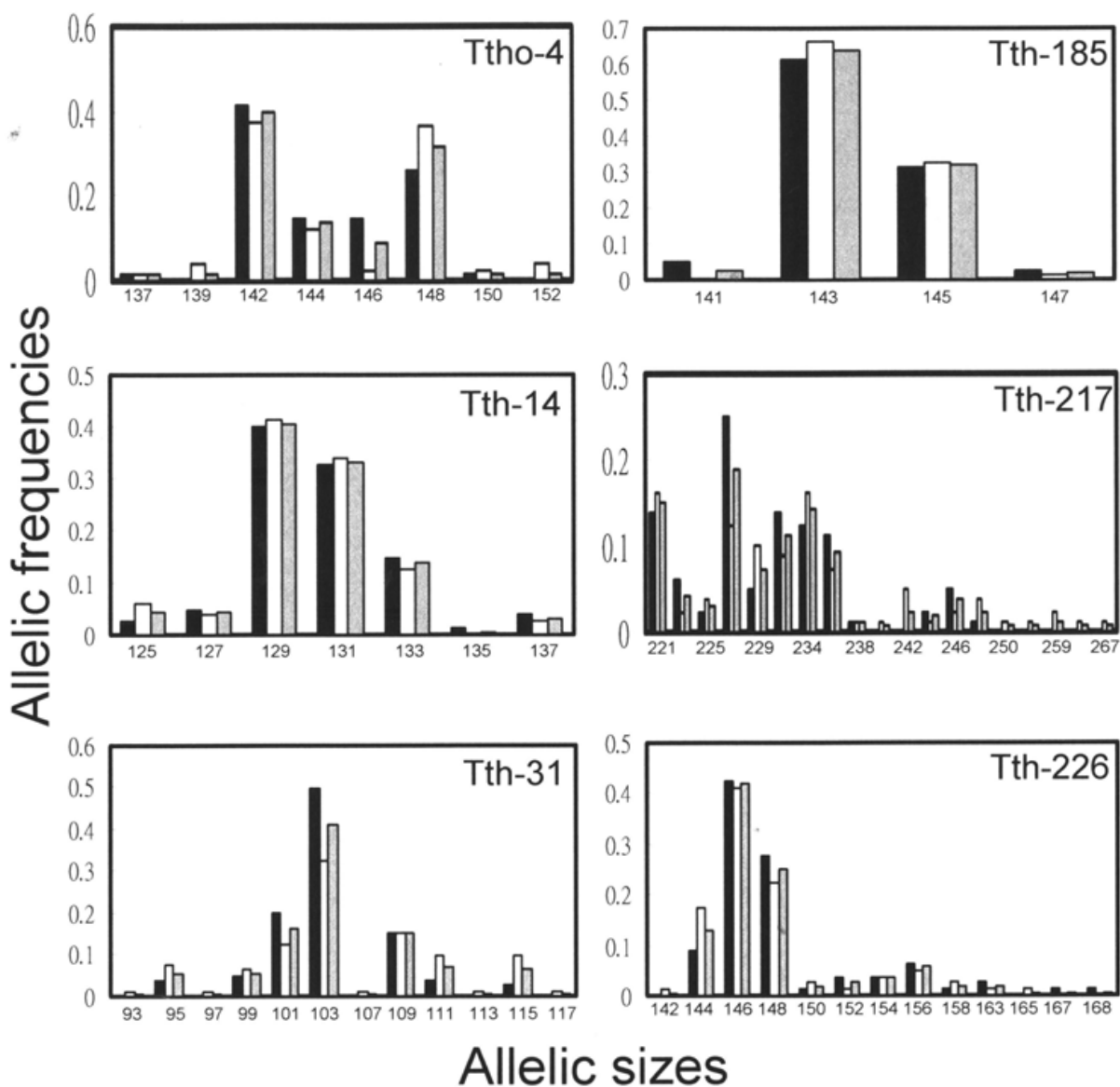


Fig. 7. Allelic sizes and frequencies from six microsatellite loci. White, Taiwanese samples; black, New Zealand samples; gray, overall sample.

## 4. Discussion

### 4.1 Interspecific divergence

In the past, tuna species identification was attempted using several different nuclear and mitochondrial markers. However, those results produced various conclusions, until Viñas & Tudela (2009) used a validated methodology for genetic identification of *Thunnus* species. They indicated that the combination of two genetic markers, one mitochondrial *CR* and another nuclear *ITS 1*, allowed full discrimination among all eight tuna species. In this study, we found that the *Cyt b* gene is also a well-established marker to distinguish *Thunnus* species, and it has been widely used in taxonomic studies of marine fishes in general (Johns & Avise, 1998). In a previous report, Block et al. (1993) constructed a phylogeny of tunas, bonitos, mackerels, and billfish based on a partial *Cyt b* gene sequence (about 515 bp in



Samples Loci	Taiwan sample N = 40	New Zealand sample N = 40	Overall N = 80
<b>Ttho-4</b>			
HWE test	n.s.	n.s.	n.s.
na/ne	6/3.517	8/3.426	8/3.566
I	1.395	1.481	1.495
H <sub>O</sub>	0.6	0.750	0.675
H <sub>E</sub>	0.725	0.717	0.724
<b>Tth14</b>			
HWE test	n.s.	n.s.	n.s.
na/ne	7/3.415	6/3.272	7/3.35
I	1.436	1.38	1.419
H <sub>O</sub>	0.7	0.675	0.688
H <sub>E</sub>	0.716	0.703	0.706
<b>Tth31</b>			
HWE test	n.s.	*	*
na/ne	7/3.140	12/5.745	12/4.27
I	1.441	2.012	1.785
H <sub>O</sub>	0.575	0.55	0.563
H <sub>E</sub>	0.690	0.836	0.771
<b>Tth185</b>			
HWE test	n.s.	*	*
na/ne	4/2.101	3/1.836	4/1.965
I	0.906	0.693	0.818
H <sub>O</sub>	0.425	0.200	0.313
H <sub>E</sub>	0.531	0.461	0.494
<b>Tth217</b>			
HWE test	n.s.	n.s.	n.s.
na/ne	12/7.191	19/10	19/8.945
I	2.165	2.544	2.435
H <sub>O</sub>	0.725	0.75	0.738
H <sub>E</sub>	0.872	0.911	0.894
<b>Tth226</b>			
HWE test	n.s.	n.s.	n.s.
na/ne	11/3.675	11/3.888	13/3.824
I	1.663	1.682	1.705
H <sub>O</sub>	0.775	0.75	0.763
H <sub>E</sub>	0.737	0.752	0.743
Total no. of alleles	47	59	63
Mean I per locus	1.501 ± 0.410	1.632 ± 0.624	1.609 ± 0.529
Mean na per locus	7.833 ± 3.833	9.833 ± 5.565	10.50 ± 5.32
Mean ne per locus	3.840 ± 1.735	4.695 ± 2.888	4.32 ± 2.396
Mean H <sub>O</sub> per locus	0.633 ± 0.127	0.613 ± 0.217	0.623 ± 0.167
Mean H <sub>E</sub> per locus	0.712 ± 0.109	0.730 ± 0.154	0.722 ± 0.130

\* Significant at the 5% level; n.s., not significant.

Table 3. All information of *I* (Shannon index), *na* (observed allelic numbers), *ne* (effective allelic numbers), *H<sub>O</sub>*, *H<sub>E</sub>*, and test of Hardy-Weinberg equilibrium (HWE). \* Indicates a significant deviation from HWE.

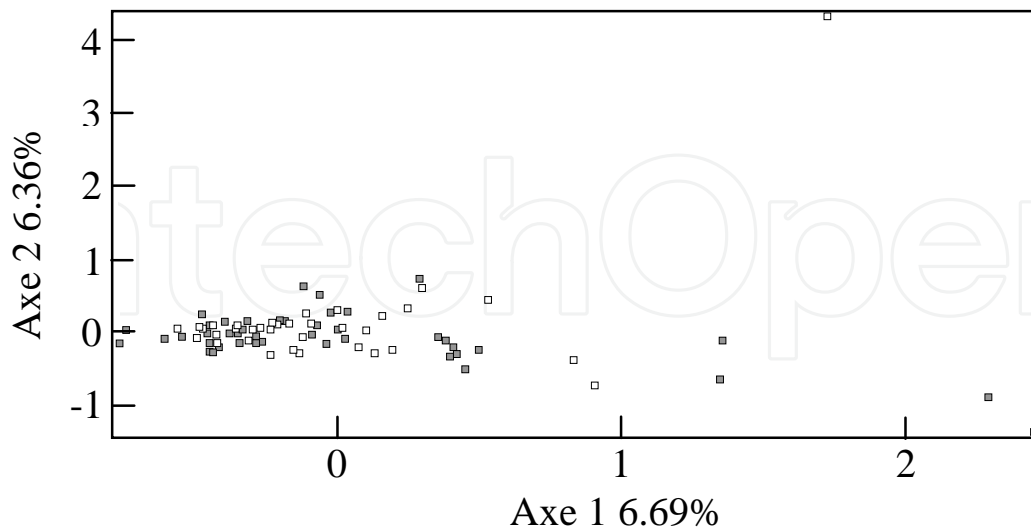


Fig. 8. Distribution of the factorial correspondence analysis between two Pacific bluefin tuna specimens from Taiwan and New Zealand waters. Gray squares, specimens from Taiwan; white squares, specimens from New Zealand waters.

length). The marker supplied important evolutionary information among these Scombridae fishes. Tseng et al. (2011) successfully distinguished the three bluefin tunas *T. orientalis*, *T. maccoyii*, and *T. thynnus* using the full-length *Cyt b* gene (1141 bp). Consequently, for this study, the entire *Cyt b* gene sequence could identify all eight *Thunnus* species.

Some specific nucleotide sites are valid for species identification of *Thunnus* tunas. Moreover, all eight subcloned and seven reference sequences had significant clusters among species, suggesting that the *Cyt b* gene is a useful marker for distinguishing *Thunnus* species. However, the identification of hybrid individuals is still difficult when a single genetic marker, such as *Cyt b*, is used. This implies that when one needs to verify equivocal tuna specimens, both nuclear and mitochondrial DNA should simultaneously be applied for identification.

*Thunnus* phylogeny was clarified on the NJ tree of the *Cyt b* gene. Two significant groups were present on the NJ tree with high bootstrapping values. Results indicated that *T. alalunga* and *T. orientalis* are sister species and share a recent common maternal ancestor, which is consistent with previous reports from Alvarado-Bermer et al. (1997), Ward (1995), and Chow & Kishino (1995). *Thunnus thynnus* and *T. maccoyii* have a closer relationship as do *T. atlanticus* and *T. albacares* with high bootstrapping value support in the NJ tree. Moreover, the NJ tree supports monophyly for the tropical yellowfin group, but not for the temperate bluefin group. This is similar to results from Elliott & Ward's (1995) report which indicated that some bluefin tunas were much closer to the yellow tuna than to albacore and bigeye tunas. In summary, these data suggest that the phylogeny of *Thunnus* species does not fit into the current two-subgenera *Thunnus* and *Neothunnus* classification pattern.

Block et al. (1993) used a partial sequence of the *Cyt b* gene to draw an NJ tree and suggested that *T. alalunga* is the presumed most primitive species. In our study, two groups in the *Cyt b* genealogy seemed to have evolved in parallel. It is difficult to infer which species is the most ancestral species in *Thunnus* according to our NJ tree. The difference between a previous report by Block et al. (1993) and our results may have resulted from various sampling and sequence completeness. However, in this study we sequenced the entire *Cyt b* gene from all *Thunnus* species. Chow et al. (2003) used the mtDNA ATCO sequence to analyze *Thunnus* phylogeny and suggested that *T. alalunga* and *T. orientalis* should be sister species which was consistent with our results. According to these results, we suggest that bluefin tunas had a more-divergent origin.

#### 4.2 Intraspecific divergence

The mtDNA *CR* of *T. orientalis* was AT-rich and similar to those of many other fishes. Short tandem repeat sequences were reported in the *CR* of some fishes (Ishikawa et al. 2001), but not in *Thunnus* species, except in *T. orientalis* (Alvarado-Bremer et al. 1997). In *T. orientalis*, double repeated sequences were present in 6/40 specimens and indicated that tandem repeat sequences may be a derived character in *Thunnus* species. Most of the central haplotypes in the MST were from sample T, while most individuals from sample Z were located at the terminus. Most of the haplotypes were located at the apex of the tree which is considered to be the result of adaptive radiation.

There is plenty of evidence to show a lack of differentiation in *T. orientalis* between Taiwanese and New Zealand waters. For one thing, the genetic distance of *CR* between the T and Z samples was equal to the mean genetic distances within samples. What is more, the *CR* had a lower *Fst* between the two samples with an insignificant probability. One final point, the NJ topology for the *CR* did not show a clear geographical grouping, and all haplotypes from samples T and Z were scattered within the NJ tree and MST. It should be concluded, from what was said above, that the null hypothesis of specimens from Taiwanese and New Zealand waters being taken from a single population cannot be rejected.

The mean  $H_O$  and  $H_E$  values were very similar between the T and Z samples. Low and non-significant genetic differentiation indices of *Fst* and *Rst* between these two samples indicated that individuals of *T. orientalis* belong to the same population, and no individuals could be assigned to a specific group according to the factorial correspondence analysis. Both mtDNA and microsatellite results led to the conclusion that the lack of genetic differentiation between the T and Z samples of *T. orientalis* is consistent with a “one-stock” hypothesis. In other tuna species DNA studies have revealed little intra-specific divergence within, but significant divergence between ocean basins. In bigeye tuna (*T. obesus*) DNA markers showed no divergence among population samples from the western Pacific Ocean (Chiang et al., 2006), but high divergence among Atlantic and Indo-Pacific populations (Chow et al., 2000; Chiang et al., 2006; 2008; Martinez et al., 2006). Similarly, in albacore tuna (*T. alalunga*) no genetic differences were detected among samples from the Northwest Pacific Ocean (Wu et al., 2009), but significant differences were reported among Atlantic and Indo-Pacific populations (Vinas et al., 2004). In the other bluefin tuna no spatial genetic structure was detected among samples of southern bluefin tuna (*T. maccoyii*) from the Indian

and Pacific Oceans (Grewe et al., 1997), as expected for a species with a single spawning ground in the Java Sea. In contrast, in the Atlantic bluefin tuna (*T. thynnus*) no spatial genetic heterogeneity was detected among samples in the eastern Atlantic Ocean (Pujolar et al., 2003), but populations from the Gulf of Mexico and the Mediterranean were genetically distinct (Boustany et al., 2008). In recent decades, many wild organisms have experienced many environmental and harvesting effects that have led to population declines. For example, the abundance of juveniles of the freshwater eel *Anguilla japonica* Temminck & Schlegel, 1846 has decreased since 1970 due to climate change and overfishing (Dekker, 2003). The spawning stock of Atlantic bluefin tuna in the western Atlantic declined by ~50% between 1970 and 2000 (Porch, 2005). Southern bluefin tuna *T. maccoyii* is now in a depleted state. Historically, the stock was exploited for more than 50 years, with total catches peaking at 81,750 tons in 1961. However, the spawning stock was estimated to be around 10,000~20,000 tons in the previous 10~20-year period with a historically and critically low level (CCSBT, 2010). Currently Pacific bluefin tuna exhibits a high level of genetic polymorphism and population states under mutation-drift equilibrium, but significant catch decreases have occurred in the last decade due to overexploitation. Therefore, it is necessary to monitor the genetic diversity of the Pacific bluefin tuna population over a long period of time in the future.

## 5. Conclusions

The *Cyt b* gene is a well-established marker to distinguish *Thunnus* species. The genealogy of *Cyt b* suggested that *T. orientalis* and *T. alalunga* are sister species with a high bootstrapping value and supported monophyly of the tropical yellowfin group, but not of the temperate bluefin group. The phylogeny of *Thunnus* species does not fit into the current two-subgenera *Thunnus* and *Neothunnus* classification pattern. The mtDNA CR of *T. orientalis* shows that a lack differentiation in *T. orientalis* from Taiwanese and New Zealand waters is consistent with a “one-stock” hypothesis.

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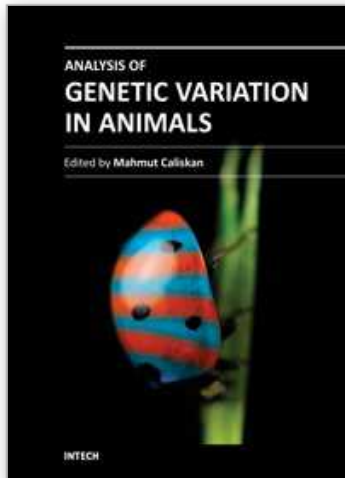


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## **Analysis of Genetic Variation in Animals**

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Analysis of Genetic Variation in Animals includes chapters revealing the magnitude of genetic variation existing in animal populations. The genetic diversity between and within populations displayed by molecular markers receive extensive interest due to the usefulness of this information in breeding and conservation programs. In this concept molecular markers give valuable information. The increasing availability of PCR-based molecular markers allows the detailed analyses and evaluation of genetic diversity in animals and also, the detection of genes influencing economically important traits. The purpose of the book is to provide a glimpse into the dynamic process of genetic variation in animals by presenting the thoughts of scientists who are engaged in the generation of new idea and techniques employed for the assessment of genetic diversity, often from very different perspectives. The book should prove useful to students, researchers, and experts in the area of conservation biology, genetic diversity, and molecular biology.

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