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Variation in the Karyotype, Cytochrome *b* Gene, and 5S rDNA of Four *Thunnus* (Perciformes, Scombridae) Tunas

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Yan-Horn Lee, Tsair-Bor Yen, Chiu-Fen Chen, and Mei-Chen Tseng (2018) *Thunnus* tunas in Scombridae are divided into the temperate subgenus *Thunnus* (bluefin group) and tropical subgenus *Neothunnus* (yellowfin group) species based on anatomic traits and distributions. The main purpose of this study was to examine the systematic status of *T. obesus* based on karyotype, cytochrome (Cyt) *b* gene, and 5S ribosomal DNA sequences. All *T. obesus*, *T. albacares*, *T. alalunga*, and *T. orientalis* specimens were caught in southeastern coastal waters off the main island of Taiwan. The karyotypical formula of *T. obesus* was 2 m + 2 st + 44 t, that of *T. albacares* was 2 m + 2 sm + 2 st + 42 t, that of *T. alalunga* was 2 m + 2 sm + 2 st + 42 t, and that of *T. orientalis* was 2 m + 2 sm + 44 t (m: metacentric; sm: submetacentric; st: subtelocentric; t: telocentric chromosome). According to a molecular genetics analysis for these species using Cyt *b* gene sequences (1141 bp), interspecific genetic distances ranged from 0.004 (*T. orientalis* vs. *T. alalunga*) to 0.038 (*T. alalunga* vs. *T. obesus*). The genealogy tree exhibited these 4 species as being categorized into 4 monophyletic groups with high bootstrapping values; *T. alalunga* and *T. orientalis* are sister species. This result suggests that the species currently allocated in *Thunnus* and *Neothunnus* might need new taxonomic characters to redefine the monophyly of the two subgenera. The sequence lengths of all cloned 5S genes from the 4 species ranged from 327-342 bp. Interspecific genetic distances ranged from 0.016 (*T. orientalis* vs. *T. alalunga*) to 0.111 (*T. orientalis* vs. *T. albacares*). The phylogenetic tree based on 5S rDNA shows *T. obesus* divided into 2 groups: one similar to *T. albacares* and the other close to *T. orientalis*. These results imply that *Thunnus* tunas have a common synapomorphic character with Scombridae fish (2n = 48) and high numbers of telocentric chromosomes (42-44). *Thunnus orientalis* and *T. alalunga* are sister based on molecular data. *Thunnus obesus* may have been derived from a more-complicated speciation processes.

Key words: *Thunnus albacares*, *T. alalunga*, *T. obesus*, *T. orientalis*, synapomorphic character.

BACKGROUND

Thunnus (Perciformes, Scombridae) tunas are highly migratory fishes that are mainly distributed in tropical and temperate oceans. Tunas are significant economic species, but are recently facing population collapse (Hutchings 2000; Fromentin and Powers 2005; MacKenzie et al. 2008). Regional tuna fishery organizations have taken management actions and set quotas

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in different countries to protect tuna resources for sustainable use. The genus *Thunnus* contains 8 species around the world, and their origins were traced back to the Middle Eocene by fossil records (Carrol 1988; Benton 1993). Collette (1978) distinguished *Thunnus* species into different subgenera, temperate *Thunnus* (the bluefin group) and tropical *Neothunnus* (the yellowfin group), based on whether or not they have a central heat exchanger. Therefore, albacore *T. alalunga*, bigeye tuna *T. obesus*, Atlantic bluefin tuna *T. thynnus*, Pacific bluefin tuna *T. orientalis*, and Southern bluefin tuna *T. maccoyii* were classified into the bluefin group, while the yellowfin group comprises blackfin tuna *T. atlanticus*, longtail tuna *T. tonggol*, and yellowfin tuna *T. albacares*. The bigeye tuna is currently classified into the bluefin group (Collette 1978) because it can adapt to cold water environments (Gibbs and Collette 1967), but there is still debate as to whether bigeye tuna should belong to the bluefin or yellowfin group because it also shares external morphological features of both groups. Therefore, this study examines genetic differences among bigeye, yellowfin tuna, albacore, and Pacific bluefin tuna, and results are used to investigate this question.

Chromosomes are the major carriers of genetic material. Many chromosomal characteristics with special changes between species can provide information for understanding the genetic variation and evolution of organisms (Takai and Izutsu 2008). Nowadays there are approximately 24,000 species of fish in the world, but only 13% have karyotypic information analyses (Klinkhardt et al. 1995). Karyotypes of *Thunnus* species were rarely studied in the past decade.

Previous studies suggest that 5S ribosomal (r) DNA sequences might be of considerable value as genetic markers to identify species, subspecies, populations, strains, and hybrids in fishes (Pendás et al. 1995; Carrera et al. 2000). A common characteristic of 5S rDNA is multiple tandemly arrayed repeats at one or several chromosomal locations throughout the genome, and 5S rDNA was also reported to be linked to other genes or arranged as a spread of additional copies (Drouin and de Sá 1995). One repetitive unit of 5S rDNA consisted of a highly conserved 120 bp transcribed region (TR) and a variable non-transcribed spacer (NTS) sequence (Aranishi 2005). Fishes share an average similarity of 95% in their 5S TRs (Martins and Wasko 2004). Ferreira et al. (2007) showed that the 5S TR sequence was highly conserved among *Leporinus* species, whereas NTSs exhibited high levels of variation in the genome because they are free to mutate. Therefore, principal variations in NTSs owing to insertions/deletions, minirepeats, and pseudogenes have often been characterized in several organisms, and these variations might represent valuable markers for population and/or species characterization (Martins and Wasko 2004). However, nuclear 5S rDNA has never previously been studied in *Thunnus* species. The mitochondrial cytochrome *b* (Cyt *b*) gene is popularly used in systematic studies to resolve divergences at many taxonomic levels (Farias et al. 2001). Finnerty and Block (1995) explored *Thunnus* systematics using a portion of the Cyt *b* gene. However, only five of the eight tuna species were analyzed, and results were insufficient to draw conclusions about relationships within the genus *Thunnus*.

The objective of the present study was to examine interspecific differences among *T. obesus*, *T. albacares*, *T. alalunga*, and *T. orientalis* by analyzing karyotypes, whole Cyt *b* genes, and 5S rDNA sequences; moreover, the phylogeny of the *Thunnus* species and systematic state of *T. obesus* are discussed.

MATERIALS AND METHODS

Sampling

Ten specimens each of bigeye, yellowfin, albacore, and Pacific bluefin tunas (with total lengths ranging 40-54 cm) were randomly obtained from angling vessels in waters off southeastern Taiwan (Fig. 1) and transferred to the Biotechnology Division, Fisheries Research Institute in Tungkang, southern Taiwan for chromosome preparation. A piece of muscle tissue from each specimen was preserved in 95% ethanol (EtOH) and stored at the Fish Biology Laboratory in National Pingtung University of Science and Technology. Ten specimens were used for karyotype analysis, three for Cyt *b*, and three for 5S rDNA for each species.

Chromosome preparation and staining

Ten fishes of each species were anesthetized by immersing them in ice water. Chromosomes were prepared from the cephalic kidney of each specimen. After a piece of cephalic kidney tissue was excised, cells were cultured in tubes with minimum essential medium (MEM), 15% fetal

bovine serum (GIBCO, New York, NY, USA), and a mitotic inhibitor (0.0002% colchicine). These tubes then were placed on a Rota-mixer (Firstek Scientific, Taipei, Taiwan) at 100 rpm and room temperature for 2 h. After culturing was complete, tubes were centrifuged at 3000 rpm for 5 min and the supernatant was decanted. A hypotonic solution (0.075 M KCl) was added to the tubes at room temperature for 30 min. After being centrifuged (3000 rpm for 5 min), the supernatant was decanted. Cells were fixed in a freshly prepared fixative solution (methanol : acetic acid = 3 : 1) at room temperature for 15 min followed by centrifugation (3000 rpm for 5 min), and the supernatant was decanted. The fixation process was repeated three times. At the end of fixation, 0.5-1.5 mL of fixative solution was added. One or 2 drops of a homogenized cell suspension was dripped onto hot slides (40-50°C) from a height of 20-30 cm and air-dried to form a ring of cells. The slides were further stained with 5% Giemsa solution (Sigma-Aldrich, St. Louis, MO, USA) for 10 min and rinsed with distilled water. After being air-dried at room temperature, the slides were mounted with gum arabic (Ledley et al. 1972). Chromosomes were observed using an optical microscope (Leica Microsystems, Wetzlar, Germany) at 1000× with lens oil. Digital images of the chromosomes were recorded and analyzed using a chromosome band analytical system (BandView 5.5, Applied Spectral Imaging, Migdal HaEmek, Israel). Chromosomes were classified into four groups - metacentric (m), submetacentric (sm), subtelocentric (st), and telocentric (t) - according to the system described by Levan et al. (1964).

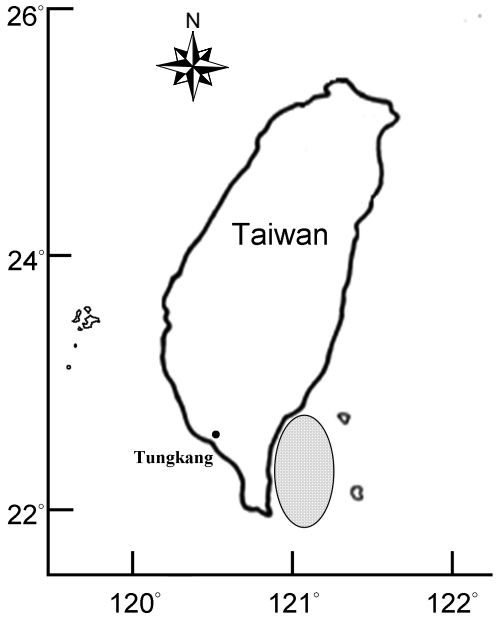

Fig. 1. Sampling locations (ellipse) of *Thunnus obesus*, *T. albacares*, *T. alalunga*, and *T. orientalis* in Taiwanese waters.

DNA isolation

Three specimens of each species were randomly selected for DNA analyses. Approximately 100 mg of muscle tissue from each specimen was put into an eppendorf tube. Before DNA purification, the tube was placed in a 60°C oven for 10 min to evaporate the EtOH. Genomic DNA was isolated using a Gentra Puregene Core kit A (Qiagen, Venlo, the Netherlands), and the purified DNA specimen was dissolved in TE buffer (1 M Tris-HCl at pH 8.0 and 0.2 mL EDTA, 0.5 M). DNA concentrations were estimated using a Nanodrop 2000C spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) with absorbance at 260 nm. The purity of a DNA preparation was checked by the ratio of absorbances at 260 and 280 nm ($A_{260}/A_{280} \geq 1.8$). DNA stock solutions were stored in a -20°C freezer.

Amplification of the Cyt *b* gene and 5S rDNA

Polymerase chain reaction (PCR) reactants contained 5 ng genomic DNA, 10 pmol each of forward and reverse primers, 4 μL 2.5 mM dNTP, 0.2 μL 25 mM $MgCl_2$, 1 U *Taq* polymerase, and 5 μL 10× buffer, and double-distilled (dd) H_2O to total 50 μL of solution. The forward and reverse primer sequences of the Cyt *b* gene were 5'-ACCAGGACTAATGGCTTG-3' and 5'-AGGATTTTAACCTCCGACGTC-3', respectively (Tseng et al. 2011). The primers used in the 5S rDNA gene amplification were forward (5'-TACGCCCGATCTCGTCCGATC-3') and reverse (5'-CAGGCTGGTATGGCCGTAAGC-3') (Martins and Galetti 1999). The PCR machine (BIO-RAD MJ Mini Gradient Thermal Cycler, Conmall Biotechnology, Singapore) was set to different conditions for the Cyt *b* and 5S rDNA amplifications. The Cyt *b* gene was amplified with initial denaturation at 95°C for 5 min; 35 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min; and a final extension of 72°C for 10 min. The reaction was cooled down to 25°C for 10 min. The 5S rDNA gene was amplified as follows: 95°C 5 min for initial denaturation; 40 cycles of 92°C

for 30 s, 64°C for 20 s, and 72°C for 30 s; with a final extension at 72°C for 5 min. The reaction was cooled down to 25°C for 10 min. PCR products of the Cyt *b* and 5S rDNA genes were checked using 1% and 1.5% agarose gel electrophoresis and then stained with ethidium bromide (EtBr; 0.5 mg/mL). Target DNA fragments were eluted with a DNA Clean/Extraction kit (GeneMark, Taichung, Taiwan). Sizes of the purified DNA fragments were checked by electrophoresis gel and then stored in a -20°C freezer.

Amplified products of the Cyt *b* gene were eluted and sequenced directly. The fragments were sequenced on an Applied Biosystems (ABI, Foster City, CA, USA) automated ABI3730x1 DNA sequencer using a Bigdye sequencing kit (Perkin-Elmer, Wellesley, MA, USA). The forward and reverse primers were used in the sequencing reaction, respectively 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. The 5S rDNAs were ligated to the pGEM-T-Easy Vector (Promega, Madison, WI, USA). The ligation products were transformed into competent cells of the *Escherichia coli* JM109 strain. After transformation, all cells were cultured on an LB plate with X gal, IPTG, and ampicillin (100 mg/ mL) and then incubated at 37°C overnight. Target colonies were selected by a blue-white screening method, and PCR product sizes were determined using Sp6 and T7 primers. Two to four target colonies for each specimen were incubated in 5 mL of LB broth with ampicillin (100 mg/ mL) overnight. Plasmids were isolated using a Plasmid Miniprep Purification kit (GeneMark, Taichung, Taiwan). Plasmid DNA was checked by gel electrophoresis with EtBr staining. The inserted fragments were sequenced following the above description.

Sequence analysis

In total, 12 Cyt *b* and 36 5S rDNA sequences were obtained. Homologous sequences were aligned using ClustalW (Thompson et al. 1994) and then manually checked. Interspecific and intraspecific genetic distances were analyzed using the Kimura-2-parameter (K2P) model (Kimura 1980), and numbers of different nucleotides were calculated using MEGA 6 software (Tamura et al. 2013). The best-fitting models of DNA substitution were determined using the lowest Bayesian Information Criterion (BIC) scores (5616.933) (Posada and Buckley 2004) as the HKY + G model. The genealogy trees of Cyt *b* sequences were constructed using the Neighbor-joining (NJ) (Nei and Kumar 2000) and Maximum-likelihood (ML) methods (Sullivan 2005). The best model for 5S rDNA substitution pattern was K2P with the lowest BIC score (3573.532). Intraspecific and interspecific K2P genetic distances and numbers of different nucleotides were calculated. The genealogy trees of 5S rDNA were reconstructed by NJ and ML methods. Cluster confidence levels of Cyt *b* and 5S rDNA were assessed using a bootstrap analysis with 1000 replications (Felsenstein 1985).

RESULTS

In cytogenetic studies, Giemsa staining of *T. obesus*, *T. albacares*, *T. alalunga*, and *T. orientalis* indicated that the diploid number of these species was 2n = 48. The karyotypic formula of *T. obesus* was 2 m + 2 st + 44 t, that of *T. orientalis* was 2 m + 2 sm + 44 t, and those of *T. albacares* and *T. alalunga* were 2 m + 2 sm + 2 st + 42 t. All *Thunnus* specimens had a high number of telocentric chromosomes with fundamental arm numbers (FNs) of 50-52 (Fig. 2, Table 1). *Thunnus albacares* (yellowfin group) and *T. alalunga* (bluefin group) have similar karyotypes. Cytogenetic characters are too conserved to distinguish these yellowfin and bluefin groups.

Table 1. List of karyotypic studies of five *Thunnus* species

Species	Karyotype	2n	FN	Reference
T. obesus	2 m + 2 st + 44 t	48	50	this study
T. albacares	2 m + 2 sm + 2 st + 42 t	48	52	Ratty et al. (1986), this study
T. alalunga	2 m + 2 sm + 2 st + 42 t	48	52	Ratty et al. (1986), this study
T. orientalis	2 m + 2 sm + 44 t	48	52	this study
T. thynnus	2 m + 2 st + 44 t 2 m + 4 st + 42 t	48	50	Ida et al. (1993)

FN, fundamental arm numbers.

Fig. 2. Karyotypes of (a) *Thunnus obesus*, (b) *T. albacares*, (c) *T. alalunga*, and (d) *T. orientalis*.

All Cyt *b* gene sequences were1141 bp for *T. obesus*, *T. albacares*, *T. alalunga*, and *T. orientalis* as well as reference sequences of *Thunnus* species (accession nos. MG017702-5) and *Katsuwonus pelamis* (accession no. DQ197958.1) (Fig. S1). Sequences were deposited in the NCBI database under the accession numbers MG017693-695 for *T. obesus*; MG017682, MG017683, and MG017686 for *T. albacares*; MG017699-701 for *T. alalunga*; and MG017673-5 for *T. orientalis*. Percentages of nucleotide compositions did not significantly differ among these *Thunnus* species, as the A+T ratios were in the range of 53.2%-53.6%. Intraspecific mean K2P genetic distances ranged from 0.003 (*T. alalunga*, 0.001-0.004) to 0.004 (*T. albacares*, 0.003-0.006), while interspecific K2P genetic distances ranged from 0.004 (*T. orientalis* vs. *T. alalunga*) to 0.043 (*T. orientalis* vs. *T. tonggol*) (Table 2). The best model of nucleotide evolution was estimated to be the HKY + G model with BIC = 5616.933. NJ and ML analyses with extremely high bootstrap support showed that *T. orientalis* and *T. alalunga* had a close phylogenetic relationship. *Thunnus obesus*, *T. albacares*, *T. tonggol*, *T. atlanticus*, *T. thynnus*, and *T. maccoyii* were placed together in the same clade with high bootstrap support (Fig. 3). The result disagreed with the theory that *Thunnus* is divided into subgenera *Thunnus* and *Neothunnus*.

5S rDNA sequences from each specimen were subcloned from these 4 *Thunnus* species to produce a consensus length of 343 nt. The sequences were deposited in the NCBI database under the accession numbers MG017636-44 for *T. orientalis*, MG017645-53 for *T. alalunga*, MG017654-62 for *T. albacares*, and MG017663-71 for *T. obesus*. Results indicated that the sites +1 to +120 bp corresponding to the transcribed region (TR) were highly conserved across the genus. The non-TR (NTR) was variable within *Thunnus*; even so, there also were some highly conserved regions across species (Figs. S2 and S3).

Lengths of 5S rDNA ranged from 326-341 nt with 32 variable sites and 16 deletion/insertion sites within *T. obesus*, 326-341 nt with 37 variable sites and 29 deletion/insertion sites within *T. albacares*, 327-339 nt with 13 variable sites and 12 deletion/insertion sites within *T. alalunga*, and 328-338 nt with 13 variable sites and 10 deletion/ insertion sites within *T. orientalis* (Table 3). Their sequences were aligned to sequences of *Scomber scombrus*, which were selected from the NCBI GenBank (accession no. AB246033.1). Intraspecific mean K2P genetic distances of 5S rDNA sequences ranged from 0.0113 ± 0.0031 (*T. alalunga*) to 0.0440 ± 0.0079 (*T. obesus*). Interspecific genetic distances of 5S rDNA sequences ranged from 0.0237 ± 0.0064 (*T. orientalis* vs. *T. alalunga*) to 0.0882 ± 0.0154 (*T. orientalis* vs. *T. albacares*). The NJ analysis with greater than an 80% bootstrapping value showed that *T. orientalis* and *T. alalunga* had a

Table 2. Numbers of different nucleotides between tuna species (upper diagonal) and Kimura-2-parameter genetic distances of nucleotide sequences (below the diagonal) from 3 cytochrome *b* sequences of each species, 1 reference sequence of 4 *Thunnus* species, and the outgroup *Katsuwonus pelamis*. Number after each tuna's abbreviation indicates specimen code

	Katsuwonus	NBT1	NBT2	NBT3	YFT1	YFT2	YFT3	BET1	BET2	BET3	LFT1	LFT2	LFT3	*T. tonggol*	*T. maccoyii*	*T. atlanticus*	*T. thynnus*
Katsuwonus		127	127	127	123	127	120	121	121	121	122	124	125	128	128	126	123
NBT1	0.122		2	5	35	39	35	39	39	39	8	12	13	45	38	41	38
NBT2	0.122	0.002		5	37	41	35	41	41	41	8	12	13	47	40	43	40
NBT3	0.122	0.004	0.004		34	38	34	38	38	38	5	9	10	44	37	40	37
YFT1	0.118	0.032	0.033	0.031		4	3	13	15	13	31	35	36	19	16	11	8
YFT2	0.122	0.035	0.037	0.034	0.004		7	17	19	17	35	39	40	23	20	15	12
YFT3	0.114	0.032	0.032	0.031	0.003	0.006		12	14	12	31	35	36	18	16	10	7
BET1	0.116	0.035	0.037	0.034	0.012	0.015	0.011		4	2	35	39	40	22	18	16	13
BET2	0.116	0.035	0.037	0.034	0.013	0.017	0.012	0.004		4	37	41	42	24	20	18	15
BET3	0.116	0.035	0.037	0.034	0.012	0.015	0.011	0.002	0.004		33	37	38	22	18	16	13
LFT1	0.117	0.007	0.007	0.004	0.028	0.032	0.028	0.032	0.033	0.03		4	5	41	34	37	34
LFT2	0.119	0.011	0.011	0.008	0.032	0.035	0.032	0.035	0.037	0.033	0.004		1	45	38	41	38
LFT3	0.12	0.011	0.011	0.009	0.032	0.036	0.032	0.036	0.038	0.034	0.004	0.001		46	39	42	39
T. tonggol	0.123	0.041	0.043	0.04	0.017	0.021	0.016	0.02	0.021	0.02	0.037	0.041	0.042		26	18	17
T. maccoyii	0.123	0.034	0.036	0.033	0.014	0.018	0.014	0.016	0.018	0.016	0.031	0.034	0.035	0.023		20	17
T. atlanticus	0.121	0.037	0.039	0.036	0.01	0.013	0.009	0.014	0.016	0.014	0.033	0.037	0.038	0.016	0.018		5
T. thynnus	0.118	0.034	0.036	0.033	0.007	0.011	0.006	0.012	0.013	0.012	0.031	0.034	0.035	0.015	0.015	0.004	

NBT, *T. orientalis*; YFT, *T. albacares*; BET, *T. obesus*; LFT, *T. alalunga*.

close relationship. As a result, *T. obesus* had 2 clear subgroups: one was closer to *T. orientalis* and *T. alalunga*, while the other was clustered with *T. albacares* (Fig. 4). The result suggested that gene duplication occurred in *T. obesus*. Therefore, it is reasonable to conjecture that interspecific hybridization may have taken place in the process of *T. obesus* speciation. Although *T. albacares* (yellowfin group) was separated from *T. orientalis* and *T. alalunga* (bluefin group) in the geneology trees in this study, *Thunnus* systematics still need to collect more data from all species in order to

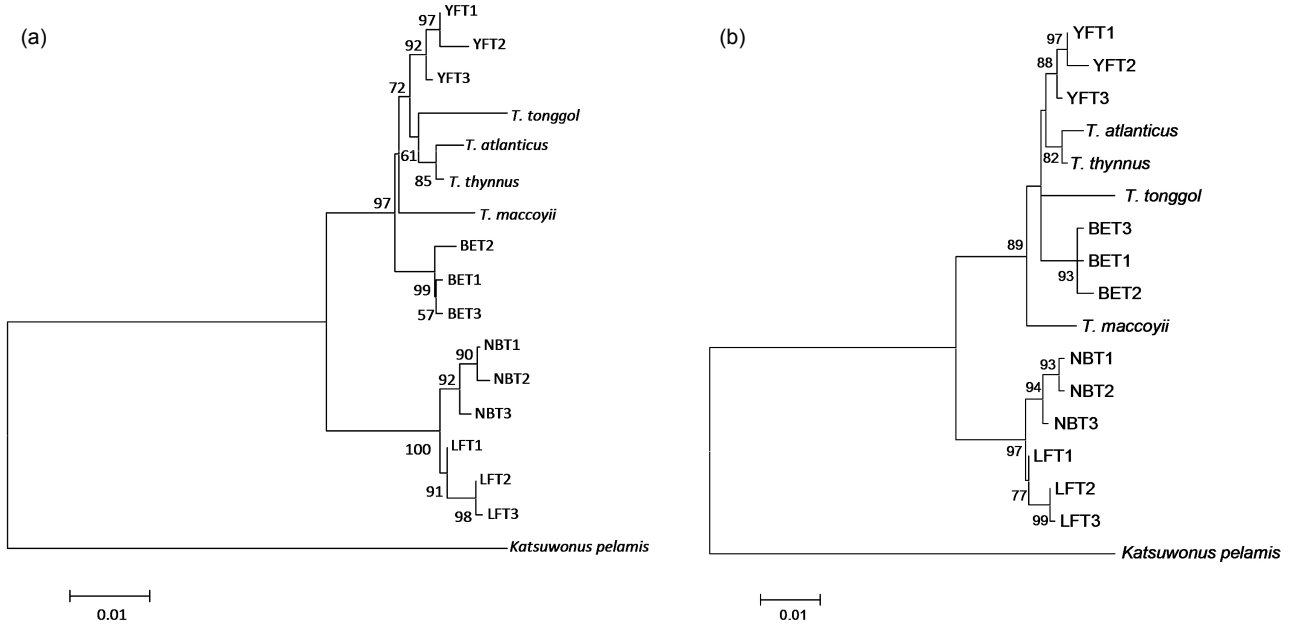

Fig. 3. (a) Neighbor-joining and (b) Maximum-likelihood trees constructed with 17 cytochrome (Cyt) *b* gene sequences from 8 *Thunnus* species and the outgroup, *Katsuwonus pelamis*. NBT: *T. orientalis*, YFT: *T. albacares*, BET: *T. obesus*, LFT: *T. alalunga*.

Table 3. Numbers of different nucleotides between tuna species (upper diagonal) and Kimura-2-parameter genetic distances of nucleotide sequences (below the diagonal) from 5S rDNAs of partial specimens of 4 *Thunnus* species and the outgroup, *Scomber scombrus*. Number after each tuna's abbreviation indicates specimen and colony codes

	Scomber	NBT 1-1	NBT 1-2	NBT 1-3	YFT 1-1	YFT 1-4	YFT 1-14	BET 2-1	BET 2-2	BET 2-3	BET 3-1	BET 3-2	BET 3-3	LFT 3-1	LFT 3-2	LFT 3-3
Scomber		124	128	122	118	125	117	123	126	123	120	121	115	127	121	125
NBT 1-1	0.616		3	5	21	20	19	16	16	15	4	2	1	6	4	5
NBT 1-2	0.646	0.012		8	24	23	22	19	19	18	7	5	4	8	6	7
NBT 1-3	0.633	0.02	0.032		26	25	24	20	20	20	9	7	6	11	9	10
YFT 1-1	0.622	0.088	0.102	0.111		9	8	9	11	8	25	23	22	21	19	19
YFT 1-4	0.607	0.084	0.098	0.107	0.036		7	6	10	5	24	22	21	20	18	18
YFT 1-14	0.612	0.079	0.093	0.102	0.032	0.028		7	9	6	23	21	20	19	17	17
BET 2-1	0.588	0.066	0.08	0.084	0.036	0.024	0.028		4	1	20	18	17	16	14	14
BET 2-2	0.614	0.066	0.079	0.084	0.044	0.04	0.036	0.016		5	20	18	17	18	16	16
BET 2-3	0.588	0.062	0.075	0.084	0.032	0.02	0.024	0.004	0.02		19	17	16	15	13	13
BET 3-1	0.65	0.016	0.028	0.036	0.106	0.101	0.097	0.084	0.083	0.079		6	5	10	8	9
BET 3-2	0.624	0.008	0.02	0.028	0.097	0.092	0.088	0.075	0.074	0.071	0.024		3	8	6	7
BET 3-3	0.605	0.004	0.016	0.024	0.093	0.088	0.084	0.071	0.07	0.066	0.02	0.012		7	5	6
LFT 3-1	0.612	0.024	0.032	0.045	0.087	0.083	0.079	0.066	0.074	0.062	0.04	0.032	0.028		2	3
LFT 3-2	0.595	0.016	0.024	0.036	0.079	0.075	0.07	0.057	0.066	0.053	0.032	0.024	0.02	0.008		1
LFT 3-3	0.595	0.02	0.028	0.04	0.079	0.075	0.07	0.057	0.066	0.053	0.036	0.028	0.024	0.012	0.004	

NBT, *Thunnus orientalis*; YFT, *T. albacares*; BET, *T. obesus*; LFT, *T. alalunga*.

complete the analysis.

DISCUSSION

The conserved diploid number of 2n = 48 was observed in the Scombridae, *e.g.*, *Auxis thazard* and *Katsuwonus pelamis* are 48 t, and *Scomber australasicus* and *S. japonica* are 2 st + 46 t (Murofushi and Aoki 1979; Hardie and Hebert 2004). Hence, 2n = 48 should be a synapomorphic character of Scombridae fish; moreover, those studies showed that Scombridae fish have high numbers (42-46) of telocentric chromosomes. In this study, 4 *Thunnus* species - *T. obesus*, *T. albacares*, *T. alalunga*, and *T. orientalis* - presented this ancestral and presumably primitive characteristic (2n = 48). *Thunnus* species also had high numbers (42-44) of telocentric (t) chromosomes, and small fundamental arm numbers (FNs) that ranged from 50-52. The karyotype formula of *T. alalunga* in this study was identical to that in a report by Ida et al. (1993) and was the same as that of *T. albacares*. The karyotype formula of *T. obesus* differed from those of *T. orientalis* and *T. albacares*, but was very similar to that of *T. thynnus* (Ratty et al. 1986). In total, 3 distinct karyotypes were observed in *Thunnus* tunas as follows: 2 m + 2 sm + 44 t, 2 m + 2 sm + 2 st + 42 t, and 2 m + 2 st + 44 t. Thus, the similarity of the karyotypical formulae in *Thunnus* tunas (Table 1) suggests that the chromosomal numbers and karyotypes may be too conserved to distinguish taxa. However, intraspecific karyotype

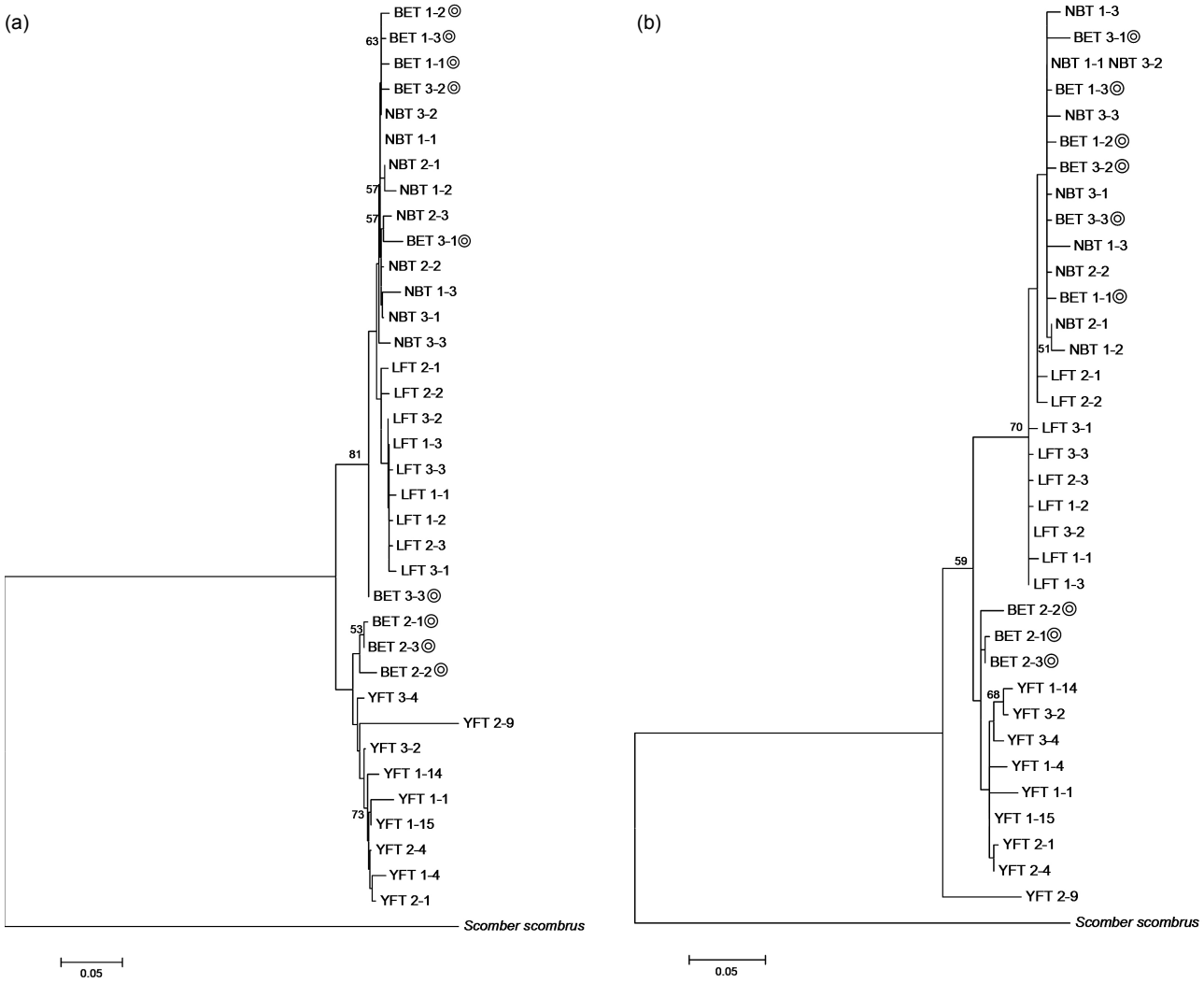

Fig. 4. (a) Neighbor-joining and (b) Maximum-likelihood trees constructed using 5S ribosomal DNA sequences from 4 *Thunnus* species and the outgroup, *Scomber scombrus*. NBT: *T. orientalis*, YFT: *T. albacares*, BET: *T. obesus*, LFT: *T. alalunga*. ◎indicates two subgroups of *T. obesus.*

polymorphism of *T. obesus* was observed in the study, *e.g*., the karyotype 2 m + 2 sm + 2 st + 42 t was also recorded in a few cells.

In the past, interspecific genetic differences among *Thunnus* species were explored using several different nuclear and mitochondrial genetic markers, *e.g*., first internal transcribed spacer (*ITS1*) of rDNA, mitochondrial ATCO, the control region, and the Cyt *b* gene. Those studies reached different conclusions. Finnerty and Block (1995) explored *Thunnus* systematics using the mitochondrial Cyt *b* gene of 5 species, and the results did not fully clarify interspecific genetic relationships. Alvarado-Bremer et al. (1997) constructed a *Thunnus* phylogeny using the mitochondrial control region portion. Their results supported the subgenera *Thunnus* and *Neothunnus* each being monophyletic, but excluded the bigeye tuna because it was difficult to classify into any subgenus. Therefore, bigeye tuna seems to have a higher similarity with tropical tunas based on an allozyme analysis (Sharp and Pirages 1978; Elliott and Ward 1995). On the other hand, Chow et al. (2006) examined interspecific nucleotide variations *ITS* sequences and suggested that introgression may have occurred between tuna species. Those results refute *Thunnus* being classified into the 2 subgenera, *Thunnus* and *Neothunnus*. In addition, a flanking region (ATCO) between the mitochondrial (mt) DNA ATPase and cytochrome oxidase III genes and *ITS1* sequences supported the yellowfin group being monophyletic, but the bluefin group possibly not. However, *T. thynnus* and *T. orientalis* had distinct mtDNA, but shared almost identical *ITS1* sequences. These molecular data suggest that intermittent speciation events occurred in species of *Thunnus* (Chow et al. 2006). Consequently, relationships among closely related *Thunnus* tunas remain unresolved. Viñas and Tudela (2009) combined 2 genetic markers, the mitochondrial control region and nuclear *ITS1*, to fully distinguish all 8 *Thunnus* species. They set up a useful molecular tool for *Thunnus* tuna identification; nevertheless, the complete Cyt *b* gene is also a well-established marker for discriminating *Thunnus* species, and it has been widely used in taxonomic studies of marine fishes in general (Johns and Avise 1998). Díaz-Arce et al. (2016), on the other hand, conducted a genome-wide nuclear marker-based phylogeny of tunas using restriction site-associated DNA sequencing data and supported that *T. obesus* should be included within the subgenus *Neothunnus*; this disagrees with the previous evolutionary history of the *Neothunnus* described by Alvarado-Bremer et al. (1997).

In this study, the Cyt *b* gene sequences could effectively differentiate 4 species: *T. obesus*, *T. albacares*, *T. alalunga*, and *T. orientalis*. When reference sequences of 4 other *Thunnus* species - *T. toggol*, *T. atlanticus*, *T. maccoyii*, and *T. thynnus* - were added to the phylogenetic analysis, the results showed that *T. orientalis* and *T. alalunga* are sister, which is consistent with results of previous studies (Chow and Kishino 1995; Ward 1995; Alvarado-Bermer et al. 1997; Chow et al. 2003). Block et al. (1993) used partial sequences of the Cyt *b* gene to map phylogenetic trees, and speculated that the albacore tuna was the most primitive species. According to the phylogenetic tree of *Thunnus* from this study, it is difficult to infer which species of tuna is the most primitive. *Thunnus obesus* was previously classified into the bluefin group (Collette 1978), but in this study *T. obesus*, *T. thynnus*, and *T. maccoyii* were clustered with all 3 yellowfin tunas in the phylogenetic tree with high bootstrapping value support. The contradictory results on the subdivision of *Thunnus* and *Neothunnus* suggest that the bluefin group involved a more-complicated evolutionary process. The use of a single genetic marker may still be insufficient to wholly identify species with a complicated evolutionary history. To overcome this weakness in the future, both nuclear and mitochondrial DNA should be used rather than using a single genetic marker.

In addition, TRs of *Thunnus* species were conserved among the analyzed sequences with just several base substitutions. The conservation implies that their function is important, *i.e*., the internal control region of the 5S TR sequence, which consists of an A box, an intermediate element, and a C box, is active as a transcription promoter (Pieler et al. 1987; Veldhoen et al. 1994), and hence the 5S TR does not represent an appropriate genetic marker to reconstruct a phylogeny because its fragment is highly conserved and short (120 bp). The NTSs of 4 *Thunnus* tunas ranged from 206-221 bp in length. Variation in the NTSs of *Thunnus* species includes substitutions and indels of 1-2 nt and short-fragment indels (4-15 bp), which suggests that extensive length variations occurred at intra-individual, intraspecific, and interspecific levels of genetic polymorphism. The NTSs also had some conserved regions across species and seem to have no function. But it was recently demonstrated that the presence of conserved elements within

the NTS play an important role in regulating the 5S TR (Hallenberg and Frederiksen 2001). In this study, the 5S rDNA of *T. obesus* and *T. albacares* appeared to have higher genetic divergences than that of the others. The genealogy results showed that *T. orientalis* and *T. alalunga* are sister, and this is consistent with the above-mentioned phylogenetic trees of Cyt *b* (Figs. 3 and 4).

Alvarado-Bremer et al. (2005) speculated that the mt control region of *T. alalunga* has genetically introgressed into that of *T. orientalis*. However, evidence of introgression was not observed in this study, which may be due to the small sample size. On the other hand, *T. albacares* can be clearly distinguished from *T. orientalis* and *T. alalunga*. We may reasonably conclude that *Thunnus* tunas can be divided into 2 groups (Figs. 3 and 4). In contrast to *T. albacares*, *T. alalunga*, and *T. orientalis*, according to 5S rDNA sequences, *T. obesus* was separated into 2 subgroups, as 6 sequences were clustered with *T. orientalis* sequences and 3 other sequences were grouped with *T. albacares* sequences (Fig. 4). In brief, the major evolutionary mechanisms of 5S rDNA duplications (Magadum et al. 2013) within *Thunnus* species were observed to be substitutions and indels. Consequently, gene duplication of 5S rDNA occurred within *T. obesus* and may have resulted from parallel evolution, convergence, or introgression. Collette (1978) indicated that *T. obesus* shares external morphological features of both bluefin and yellowfin tunas. Results of the 5S rDNA sequences also suggests that *T. obesus* shares genetic characters of both bluefin and yellowfin tunas. Hence, 2 different groups of 5S rDNA were observed within *T. obesus*, and it can be speculated that introgression occurred in the process of speciation. Alvarado-Bremer et al. (1997) proposed that *T. obesus* cannot be definitively classified into the bluefin or yellowfin group, which may be related to the high genetic diversity of *T. obesus*. *Thunnus obesus* is extensively found from the surface to deep ocean at temperatures ranging 13-29°C in the Atlantic, Indian, and Pacific Oceans (Collette and Nauen 1983). The population structure of *T. obesus* was explored, *e.g.*, by Alvarado-Bremer et al. (1997) who genetically distinguished bigeye populations between the Atlantic Ocean and the Indian and Pacific Oceans by a PCR-restriction fragment length polymorphism analysis of mitochondrial control region sequences. In addition, Chow et al. (2000) clearly indicated that not only gene flow but also fish migration between the Atlantic and Indian Oceans are severely restricted, and these distinct stocks only intermingle around South Africa. It can be concluded from the above discussion that bigeye tunas have large morphological and genetic polymorphisms, which were caused by a combination of environmental adaptations, introgressions, and a complicated process of speciation. However, sequences of these two genes of *T. obesus* from Atlantic and Indian Oceans still need to be added into the phylogenetic analysis before its species status can be properly justified.

CONCLUSIONS

Thunnus obesus, *T. albacares*, *T. alalunga*, and *T. orientalis* presented 2n = 48, a presumably primitive characteristic of Scombridae. *Thunnus* species had high numbers (42-44) of telocentric chromosomes. A phylogeny analysis of *Thunnus* tunas with more than 95% bootstrap support showed that *T. orientalis* and *T. alalunga* are sister species. The result of 5S rDNA analysis also suggests that gene duplication occurred in *T. obesus*. One duplicate was closer to *T. orientalis* and *T. alalunga*, while the other was clustered with *T. albacares*. 5S rDNA duplications may have resulted from parallel evolution, convergence, or hybridization. In conclusion, phylogenetic analyses using the Cyt *b* gene revealed that the species in these clades differ from those originally classified in the subgenera *Thunnus* and *Neothunnus*. Further genetic investigations are needed before applying new taxonomic characters to define the monophyly of the two subgenera. Larger morphological and genetic polymorphisms of *T. obesus* may be caused by a combination of environmental adaptations, introgressions, and a complicated process of speciation.

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Authors' contributions: Yan-Horn Lee designed the study and was responsible for collecting specimens as well as the chromosome preparation. Tsair-Bor Yen performed the microphotography, chromosome analyses, and statistical analyses. Chiu-Fen Chen assisted with PCR and gene cloning. Mei-Chen Tseng performed DNA-related

analyses and chromosomal analyses and wrote the manuscript. Yan-Horn Lee, Tsair-Bor Yen, Chiu-Fen Chen, and Mei-Chen Tseng participated in revising the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials: The key datasets: Cyt *b* genes of the cloned sequences of *T. obesus*, *T. albacares*, *T. alalunga*, and *T. orientalis* were deposited in the publicly available NCBI gene bank (Accession nos. MG017693-695 for *T. obesus*; MG017682, MG017683, and MG017686 for *T. albacares*; MG017699-701 for *T. alalunga*; and MG017673-5 for *T. orientalis*). 5S rDNA sequences of these 4 *Thunnus* were also deposited in the NCBI (Accession nos. MG017636-44 for *T. orientalis*, MG017645-53 for *T. alalunga*, MG017654-62 for *T. albacares*, and MG017663-71 for *T. obesus*). Other data are available in the publication.

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Supplementary Materials

Fig. S1. All variable sites within cytochrome (Cyt) *b* sequences from 12 *Thunnus* specimens and 4 published *Thunnus* sequences (Tseng et al. 2012). NBT: *T. orientalis*, YFT: *T. albacares*, BET: *T. obesus*, LFT: *T. alalunga*. (download)

Fig. S2. (A) The structure of 5S ribosomal (r) DNA containing the transcribed region (TR) and a variable non-transcribed spacer (NTS). (B) The gray region indicates variable sites, and the yellow region is the TR. NBT: *T. orientalis*, YFT: *T. albacares*, BET: *T. obesus*, LFT: *T. alalunga*. The first and second arabic numerals are codes of individual and colony, respectively. (download)

Fig. S3. All variable sites within 36 5S rDNA sequences subcloned from 12 *Thunnus* specimens. The first and second arabic numerals are codes of individual and colony, respectively. NBT1-1 and NBT3-2 shared the identical sequence. (download)