Journal of Fish Biology (2006) **68** (Supplement A), 24–35
doi:10.1111/j.1095-8649.2005.00945.x, available online at http://www.blackwell-synergy.com

Phylogenetic relationships among *Thunnus* species inferred from rDNA ITS1 sequence

S. Chow*†, T. Nakagawa‡, N. Suzuki§, H. Takeyama‡ and T. Matsunaga‡

**National Research Institute of Fisheries Science, 6-31-1 Nagai, Yokosuka 238-0316, Japan, ‡Department of Biotechnology, Tokyo University of Agriculture and Technology, Koganei 184-8588, Japan and §National Research Institute of Far Seas Fisheries, Shizuoka 424-8633, Japan*

(Received 21 October 2004, Accepted 11 August 2005)

Intra and interspecific nucleotide sequence variation of rDNA first internal transcribed spacer (ITS1) was analysed using all eight species of the genus *Thunnus* plus two out-group species within the same family, skipjack tuna *Katsuwonus pelamis* and striped bonito *Sarda orientalis*. Intraspecific nucleotide sequence variation in ITS1, including intra-genomic variation, was low, ranging from 0·003 to 0·014 [Kimura's two parameter distance (K2P)], whereas variation between species within the genus *Thunnus* ranged from 0·009 to 0·05. The Atlantic and Pacific northern bluefin tunas *Thunnus thynnus thynnus* and *Thunnus thynnus orientalis*, recently proposed to be distinct species, were found to share nearly identical ITS1 sequences (mean K2P = 0·006) well within the range of intraspecific variation. The northern bluefin tuna appeared to be a sister group to albacore *Thunnus alalunga*, with all other *Thunnus* species in a distinct clade. The ITS1 phylogeny was consistent with mtDNA phylogeny in clustering the three tropical *Thunnus* species (*T. albacares*, *T. atlanticus* and *T. tonggol*). Southern bluefin *Thunnus maccoyii* and bigeye *Thunnus obesus* tunas showed a closer affinity to this tropical tuna group than to the northern bluefin tuna and albacore. The molecular data supported mitochondrial introgression between species and contradicted morphological subdivision of the genus into two subgenera *Neothunnus* and *Thunnus*.

Key words: mtDNA; phylogeny; rDNA ITS1; tuna.

INTRODUCTION

The genus *Thunnus* South, 1845, which includes economically important large tuna species, appears to be a monophyletic unit well-supported by both morphology and molecular data (Collette *et al*., 2001). The phylogenetic relationships among species within the genus, however, are controversial. Gibbs & Collette (1967) and Collette (1979) divided the genus into two subgenera, *Neothunnus* Kishinoue, 1923 (a tropical yellowfin tuna group) and *Thunnus* South, 1845 (a more cold water tolerant bluefin tuna group), based on morphological and ecological differences. Blackfin *Thunnus atlanticus* (Lesson), longtail

†Author to whom correspondence should be addressed. Tel.: +81 46 856 2887; Fax: +81 46 857 3075; email: chow@affrc.go.jp

Thunnus tonggol (Bleeker) and yellowfin *Thunnus albacares* (Bonnaterre) tunas (designated as BKT, LTT and YFT, respectively) are members of *Neothunnus*, and albacore *Thunnus alalunga* (Bonnaterre), bigeye *Thunnus obesus* (Lowe), northern bluefin *Thunnus thynnus* (L.) and southern bluefin *Thunnus maccoyii* (Castelnau) tunas (designated as ALB, BET, NBT and SBT, respectively) belong to *Thunnus*. These groupings, however, have been challenged by several molecular genetic analyses (Chow & Kishino, 1995; Elliot & Ward, 1995; Ward, 1995; Alvarado-Bremer *et al*., 1997; Takeyama *et al*., 2001; Chow *et al*., 2003).

Previous molecular studies have indicated that the albacore is the most divergent species suggesting the subgenus *Thunnus* is not a monophyletic group (Chow & Kishino, 1995; Ward, 1995; Alvarado-Bremer *et al*., 1997; Chow *et al*., 2003). Northern bluefin tuna has been subdivided into two sub-species, *Thunnus thynnus thynnus* (L.) in the Atlantic and *Thunnus thynnus orientalis* (Temminck & Schlegel) in the Pacific (designated as NBTA and NBTP, respectively), as these two have slight differences in morphology and the populations have been well separated geographically (Gibbs & Collette, 1967; Collette & Nauen, 1983). Chow & Kishino (1995), however, found that the mitochondrial DNA (mtDNA) sequence of the Pacific northern bluefin tuna was distinct from its Atlantic counterpart and much closer to that of albacore. They also observed that the mtDNA of the Atlantic northern bluefin tuna was more similar to that of the southern bluefin tuna, and even to the tropical tunas, than to the Pacific northern bluefin tuna.

Chow & Kishino (1995) proposed hybridization followed by interspecific transfer of mtDNA as the mechanism for sequence similarity between the Pacific northern bluefin tuna and albacore. Further findings that individuals possessing the Atlantic type of mtDNA are rare but do exist in the Pacific, and those possessing the Pacific type of mtDNA exist in the Atlantic, have made the specific relationships more complicated (Chow & Inoue, 1993; Chow & Kishino, 1995; Takeyama *et al*. 2001). According to the large genetic and slight morphological differences observed between the Atlantic and Pacific northern bluefin tunas, Collette (1999) proposed that these two geographic groups should be regarded as separate species (*T. thynnus* and *T. orientalis*). Although Collette (1999) and Collette *et al*. (2001) primarily depend on mtDNA data for advocating separation of these two tunas, they have paid little attention to the molecular evidence demonstrating that both Atlantic and Pacific haplotypes exist in both ocean basins. If horizontal transfer of mtDNA exists, mtDNA sequence data cannot be relied on alone for inferring phylogenetic relationships among these closely related *Thunnus* tunas.

Under such circumstances nuclear genetic markers such as the internal transcribed spacer (ITS) region of the ribosomal RNA gene cluster may be more suitable. The ITS region evolves rapidly but the homogenizing forces of concerted evolution and molecular drive (Arnheim, 1983; Dover, 1986) are believed to act to minimize the degree of intraspecific variation, and make the ITS region suitable for phylogenetic comparisons among closely related taxa. The ITS has been used for phylogenetic studies in a very wide variety of organisms, and also in fish systematics (Pleyte *et al*., 1992; Phillips *et al*., 1994; Domanico *et al*., 1997; Sajdak & Phillips, 1997; Booton *et al*., 1999; Huyse *et al*., 2004). Though successful in resolving conflicting trees derived from nuclear and mitochondrial

DNA data in salmonids (Pleyte *et al.*, 1992; Phillips *et al.*, 1994; Domanico *et al.*, 1997; Sajdak & Phillips, 1997) or providing new insights for complicated cichlid evolution (Booton *et al.*, 1999), little attention has been paid to the intraspecific variation of the ITS region in these studies. Moreover, it proved impossible to infer phylogenetic relationships among sand goby species from ITS sequence data because of the large intraspecific sequence variation (Huyse *et al.*, 2004). In order to assess the general utility of the ITS region for inferring phylogenetic relationships in tunas, intra and interspecific nucleotide sequence variation in the ITS1 region of all *Thunnus* tunas and two species from other genera, skipjack tuna *Katsuwonus pelamis* L. and striped bonito *Sarda orientalis* Temminck & Schlegel, 1844 (designated as SKJ and SOR, respectively) was investigated.

MATERIALS AND METHODS

TUNA SAMPLES, PCR AMPLIFICATION AND NUCLEOTIDE SEQUENCING

All tuna samples were derived from the laboratory collection of the National Research Institute of Far Seas Fisheries, Japan (Table I). A total of 46 individuals was used in this study, comprising 42 individuals of the genus *Thunnus*, two SKJ and two SOR. Of the two out-group scombrids (SKJ and SOR) used, SKJ is believed to be the closest relative of the genus *Thunnus* since it is morphologically assigned to the same tribe (Thunnini) while SOR belongs to another tribe (Sardini) (Collette, 1979).

Nucleotide sequences of a conserved primer pair (ITS1 and 5·8S) used to amplify the ITS1 region were obtained from Duke University web site (http://www.biology.duke.edu/fungi/mycolab/primers.htm). The forward primer (ITS1), 5′-TCCGTAGGTGAACCTGCGG-3′, was designed to anneal near the 3′ end of 18SrDNA, and the reverse primer (5·8S), 5'-CGCTGCGTTCTTCATCG-3′, to anneal near the 5′ end of 5·8SrDNA. ITS1 was amplified using LA *Taq* polymerase (TAKARA, Japan) with GC buffer which considerably improved amplification over standard *Taq* protocols. Initial denaturation at 96° C for 2 min, was followed by 25 cycles of amplification (denaturation at 96° C for 0·5 min, annealing at 58° C for 0·5 min and extension at 74° C for 1 min) with a final extension at 74° C for 10 min. Under these conditions, a single strong fragment was amplified in all species used. Amplified fragments were purified using GENECLEAN II (BIO101). Cloning of PCR products was performed

TABLE I. Tuna species used in this study. In the phylogenetic trees, A (Atlantic) or P (Pacific) are added to the abbreviations to indicate origin

Common name	Scientific name	Abbreviation	Origin	*n*
Yellowfin tuna	*Thunnus albacares*	YFT	Atlantic, Pacific	7
Blackfin tuna	*Thunnus atlanticus*	BKT	Atlantic	2
Longtail tuna	*Thunnus tonggol*	LTT	Pacific	3
Bigeye tuna	*Thunnus obesus*	BET	Atlantic, Pacific	11
Southern bluefin tuna	*Thunnus maccoyii*	SBT	Indian Ocean	4
Northern bluefin tuna	*Thunnus thynnus thynnus*	NBTA	Atlantic	5
	Thunnus thynnus orientalis	NBTP	Pacific	3
Albacore	*Thunnus alalunga*	ALB	Atlantic, Pacific	7
Skipjack tuna	*Katsuwonus pelamis*	SKJ	Atlantic	2
Striped bonito	*Sarda orientalis*	SOR	Pacific	2

using a pGEM-T Easy Vector System I (Promega). The nucleotide sequences of the inserted PCR products were determined by automated sequencer (Applied Biosystems model 310, U.S.A.) using a ABI Big-dye Ready Reaction kit (Perkin-Elmer Cetus, Norfolk, VA, U.S.A.) with M13 forward and reverse primers and those used in the amplification. One to four clones per individual were sequenced.

PHYLOGENETIC ANALYSIS

Sequences obtained were first aligned using the CLUSTAL W algorithm in Lasergene (DNA STAR Inc.) followed by manual editing to minimize the number of gaps while still maintaining the alignment. The data set was imported into MEGA version 2.1 (Kumar *et al*., 2001) to construct phylogenetic trees using the neighbour-joining (NJ) method based on the gamma corrected Tamura-Nei (TN) or Kimura's two parameter (K2P) distance matrices. Maximum parsimony (MP) analysis with the pair-wise or complete deletion option was used for gaps. The data were also imported into PHYML (Guindon & Gascuel, 2003) for maximum likelihood analysis (ML). In the ML analysis, all gaps between sequences were removed and Modeltest version 3.06 (Posada & Crandall, 1998) was used to select the most suitable nucleotide substitution model. During the exploration of the ML topology, all parameters of the nucleotide substitution model and the gamma shape parameter were simultaneously estimated and adjusted. In order to evaluate the reliability of each node, bootstrap analysis was carried out by PHYML using the 1000 resampling data sets produced by SEQBOOT from the PHYLIP package (Felsenstein, 1993). For comparison, phylogenetic trees based on mtDNA data were investigated using the methods described above. A flanking region between the ATPase6 and the cytochrome oxidase III genes of the mtDNA (designated ATCO) was chosen based on data obtained by Takeyama *et al*. (2001) and Chow *et al*. (2003), since this is the longest sequence (857 bp) for all *Thunnus* species available to date.

RESULTS

ALIGNMENT AND CHARACTERISTICS OF THE ITS1 SEQUENCE IN TUNAS

Of 68 clones sequenced from 46 individuals, 59 clones were found to have different nucleotide sequences. Alignment of representative sequences from nine species is shown in Fig. 1. All 59 different nucleotide sequences were submitted to DDBJ (DNA data bank of Japan) (accession numbers AB127395 to AB127404 and AB211999 to AB212047). The sequence alignment among the three genera was unclear because of the extensive nucleotide substitutions and multiple indels. Such problems, however, were minimal when aligning species within genera (Table II).

Tuna ITS1 is characterized by a high GC content ranging from 68·1% in SKJ to 73·9% in YFT and considerable length variation among species (594–656 bp). In total, 51 different sequences were obtained from 42 individuals of eight *Thunnus* species (including two sub-species of NBT), in which 134 variable sites were observed. The largest observed intra-specific length variation occurred in YFT (644–656 bp) followed by BET (638–643 bp). These length differences within species and also between species within the genus *Thunnus* were primarily due to variation in the number of nucleotide repeats (shown by asterisks in Fig. 1).

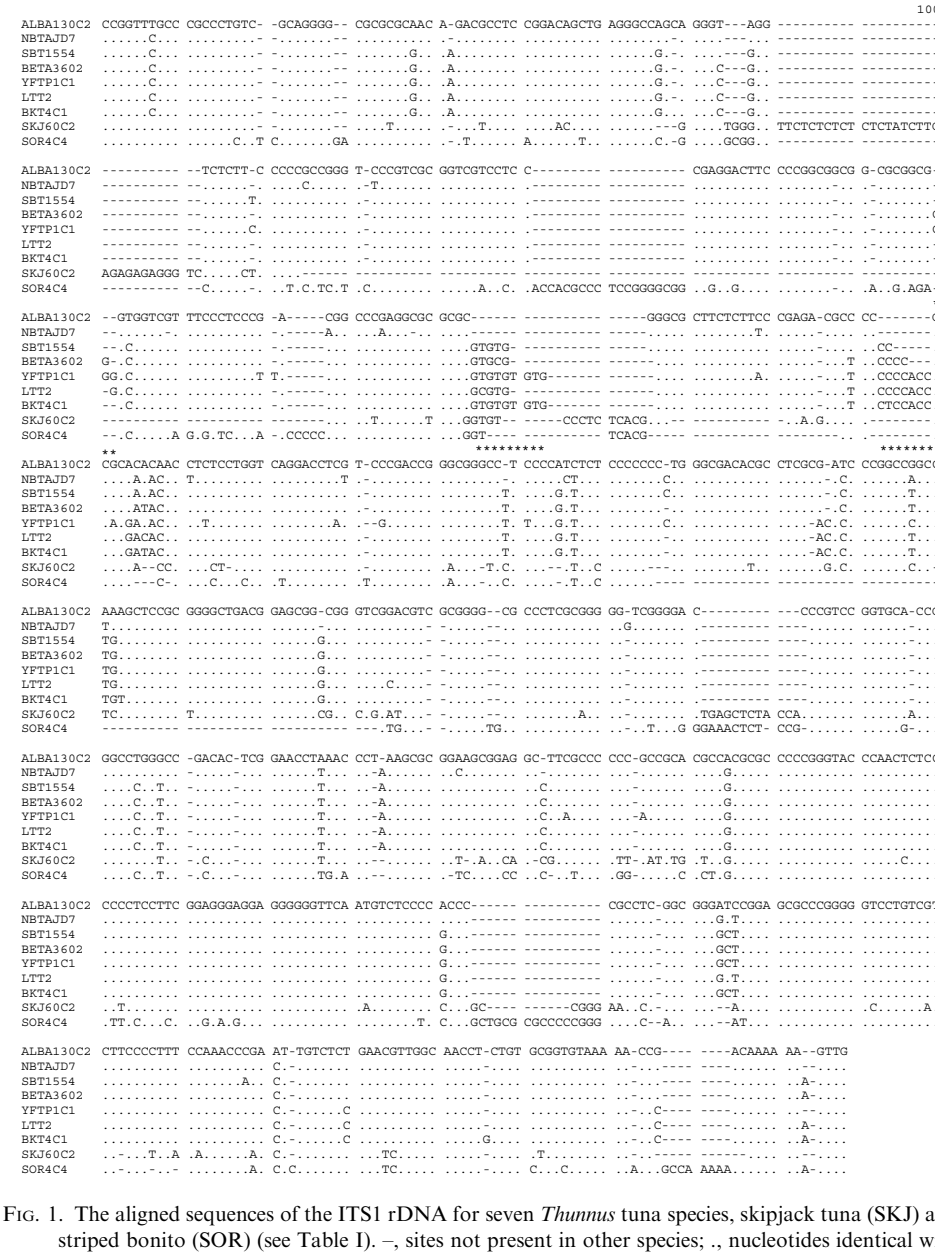

FIG. 1. The aligned sequences of the ITS1 rDNA for seven *Thunnus* tuna species, skipjack tuna (SKJ) and striped bonito (SOR) (see Table I). –, sites not present in other species; ., nucleotides identical with those of the top sequence; *, repeated nucleotides observed within the genus *Thunnus*.

To test for saturation by multiple nucleotide substitutions, per cent transitions between sequences were plotted against transversions, indicating no apparent saturation within and among the genera. The overall mean of the transition: transversion ratio (based on pair-wise deletion) was 2·05 for the total data set and 2·35 for *Thunnus* data alone.

TABLE II. Sequence information for the tuna rDNA ITS1 region

Species		Length (bp)	Per cent GC content (mean)
YFT	(13/13/7)*	644–656	72·3–73·9 (73·2)
BKT	(3/4/2)	646–649	72·8–73·4 (73·1)
LTT	(2/3/3)	644	73·4–73·8 (73·6)
BET	(12/14/11)	638–643	73·4–73·7 (73·5)
SBT	(4/4/4)	641–642	73·0–73·3 (73·2)
NBTA	(6/6/5)	627–629	71·6–72·7 (72·2)
NBTP	(2/3/3)	630	72·2–72·4 (72·3)
ALB	(9/10/7)	630–632	72·1–72·9 (72·6)
SKJ	(3/3/2)	594–597	68·1–69·0 (68·6)
SOR	(5/8/2)	608	73·0–73·2 (73·1)

* Following each species abbreviation the number of clones showing different sequences, the number of clones examined and the number of individuals are shown in parentheses.

Three clones obtained from single individuals in two species (SOR and YFT) showed different nucleotide sequences, indicating intra-genomic sequence variation.

PHYLOGENETIC ANALYSIS

Mean K2P distance within species, using the pair-wise deletion option, ranged from 0·0026 in SOR to 0·0136 in SKJ (Table III). Mean K2P distance between species within the genus *Thunnus* ranged from 0·0086 (LTT *v.* BKT) to 0·0498 (YFT *v.* ALB), whereas the differentiation between the sub-species NBTA and NBTP was small (K2P = 0·0064). Nucleotide sequence difference between genera was very large ranging from 0·1349 to 0·1652. SKJ may be a better candidate than SOR for rooting the phylogenetic tree, as SKJ is believed to be the closest relative of the genus *Thunnus* and morphologically assigned to the same tribe (Thunnini) (Collette, 1979). The ITS1 alignment between the genera, however, was equivocal and differentiation between the genera was very large. To investigate long branch attraction, four data sets were prepared comprising 1) all sequences, 2) all sequences except SKJ, 3) all sequences except SOR and 4) *Thunnus* data alone.

In ML analysis, the Hasegawa-Kishino-Yano model (Hasegawa *et al.*, 1985) with gamma correction (HKY85 + G) was selected as the best fit by Modeltest. The ML optimization under this model estimated the model parameters and inferred the tree topology. All phylogenetic trees constructed either by distance, MP or ML methods, and using all four data sets, agreed with one another in separating the genus *Thunnus* into two large clades. An NJ tree drawn using Tamura-Nei Gamma distance is shown in Fig. 2(a). NBTA and NBTP, which shared very similar sequences, appeared to be a sister group of ALB, leaving all other *Thunnus* species in a distinct clade. In contrast, in the NJ trees, poor discrimination among *Thunnus* species was obtained when only transversions were used; robustness (based on bootstrap values) of the ALB-NBT clade was

TABLE III. Mean Kimura's two-parameter distance ($\times 10^2$) (pair-wise deletion) within (on the diagonal) and between (below the diagonal) tuna species (including two sub-species of northern bluefin tuna). Standard errors are shown in parentheses. See Table I for species abbreviations

	YFT	LTT	BKT	BET	SBT	NBTA	NBTP	ALB	SKJ	SOR
YFT	1·24 (0·24)									
LTT	1·11 (0·26)	0·31 (0·22)								
BKT	1·18 (0·27)	0·86 (0·32)	0·36 (0·18)							
BET	1·71 (0·38)	1·52 (0·43)	1·49 (0·42)	0·56 (0·16)						
SBT	1·82 (0·44)	1·71 (0·50)	1·67 (0·49)	0·99 (0·31)	0·39 (0·18)					
NBTA	4·23 (0·80)	3·79 (0·79)	3·85 (0·79)	3·19 (0·69)	2·95 (0·68)	0·44 (0·16)				
NBTP	4·43 (0·78)	3·99 (0·77)	4·05 (0·77)	3·39 (0·67)	3·09 (0·66)	0·64 (0·23)	0·48 (0·27)			
ALB	4·98 (0·82)	4·66 (0·82)	4·72 (0·82)	4·04 (0·75)	3·50 (0·71)	2·91 (0·61)	2·74 (0·58)	0·48 (0·15)		
SKJ	14·93 (1·72)	14·56 (1·72)	14·50 (1·71)	13·91 (1·68)	13·49 (1·62)	13·60 (1·66)	13·59 (1·68)	14·50 (1·74)	1·36 (0·38)	
SOR	16·46 (1·86)	16·16 (1·89)	15·85 (1·87)	15·47 (1·83)	15·26 (1·80)	16·27 (1·94)	16·10 (1·94)	16·52 (1·93)	13·86 (1·77)	0·26 (0·13)

lowered and branching order varied depending on which non-*Thunnus* species were included as out-group species. All trees using *Thunnus* data alone strongly supported separation of the two clades. Thus, transitions appear to be very informative and considering only transversions is not practical when inferring relationships among *Thunnus* species. Monophyly of the three tropical tunas (BKT, LTT and YFT) was supported by the three methods, yet phylogenetic relationships among these tropical tunas were not resolved due to the large variation in YFT sequences, compounded by low sequence divergence among these tropical tuna species. BET and SBT, though morphologically and ecologically assigned to the bluefin tuna group, appeared to have greater molecular affinity with the tropical tuna species than with ALB and NBT. No phylogeographic signal was evident in the ITS1 trees for the three cosmopolitan species (ALB, BET and YFT), although genetic differentiation between the Atlantic and Pacific populations of ALB and BET has been clarified using mtDNA analysis (Chow & Ushiama, 1995; Chow *et al.*, 2000).

For comparison, phylogenetic trees using mtDNA data were investigated. Alignments of 50 sequences including all *Thunnus* species plus SKJ and SOR are available at http://www.enyo.affrc.go.jp/chow/TunaATCO.txt. The transition:transversion ratio for the *Thunnus* data set was high (8·57), and all tree topologies using the distance method (NJ) with transitions or transversions, or both, and those using MP and ML methods agreed with one another in strongly

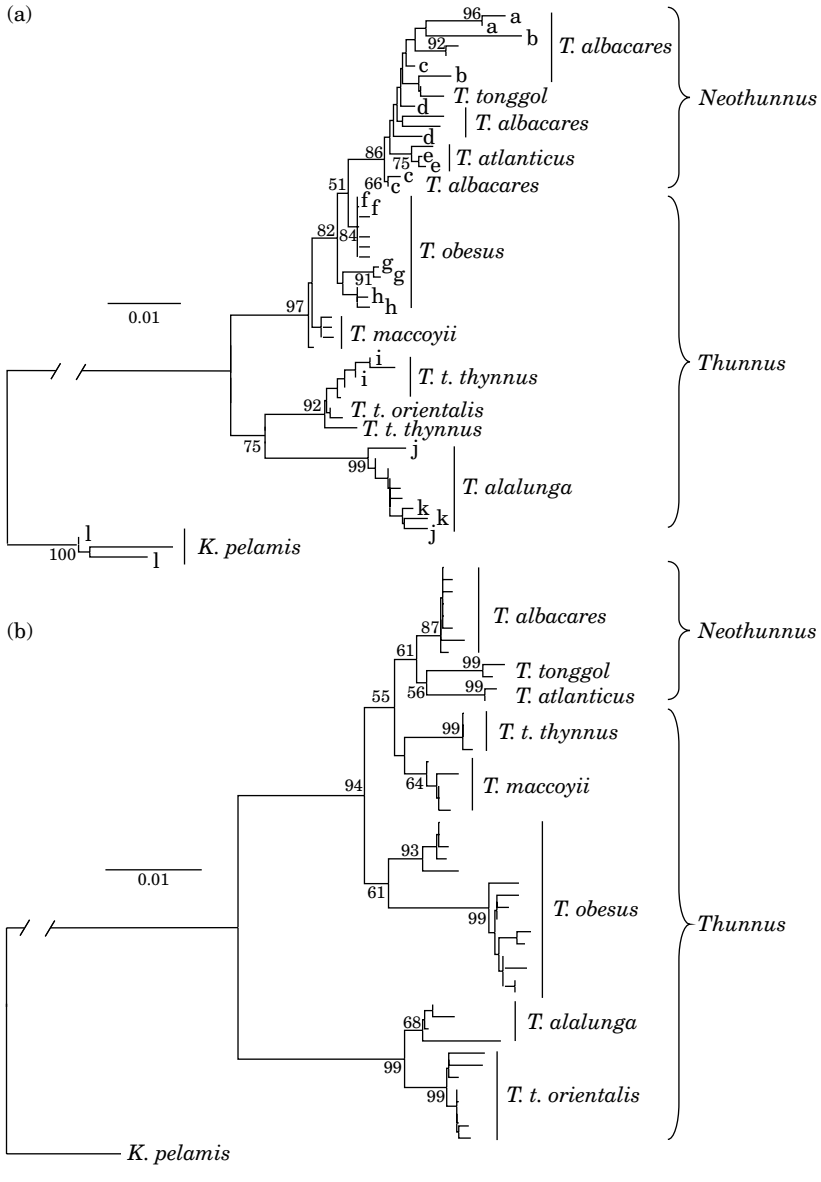

FIG. 2. Neighbour-joining phylogenetic trees constructed using the Tamura-Nei Gamma distance method based on (a) rDNA ITS1 and (b) mtDNA ATCO sequence data. Bootstrap values >50% (out of 1000 replicates) are shown at the nodes. Identical letters at the branch terminal end in the ITS1 tree indicate clones having different sequences obtained from a single individual. MtDNA sequence data were derived from Takeyama *et al.* (2001) and Chow *et al.* (2003). Note that separation between the subgenera *Neothunnus* and *Thunnus* is not supported by both trees.

supporting two large clades (ALB-NBTP and other *Thunnus*) [Fig. 2(b): only NJ tree is shown]. These two clades have been observed in the ITS1 tree, except for the position of NBTA. In the mtDNA tree, NBTA was closely related to SBT and even to the tropical tunas (BKT, LTT and YFT) and BET, while in the ITS1

tree, NBTA was most closely related to NBTP followed by ALB. Since branching order between NBTA-SBT, BE and tropical tunas varied among NJ, MP and ML trees obtained in this study and also in several investigations using different segments of the mtDNA, it remains unresolved.

DISCUSSION

Although the molecular drive or concerted evolution suggested by Arnheim (1983) and Dover (1986) may act to homogenize repeat units of rDNA, intraspecific and even intra-genomic variation in the ITS1 locus of tunas was often observed in this study. Tuna ITS1 intra-specific variation was generally lower than that between species, and intraspecific variation observed in the ITS1 of two sand goby species (*Pomatoschistus lozanoi* (de buch) and *Pomatoschistus microps* (Krøyer)) was much larger (Huyse *et al.*, 2004). The large variation in YFT combined with the sequence similarity between species obscured phylogenetic relationships among tropical tunas, indicating that depending on single sequence data from a single individual should be treated with caution. Thus, phylogenetic relationships between the closely related three tropical tunas (BKT, LTT and YFT) may not be sufficiently resolved based on analysis of the ITS1 region. Furthermore, the small number of clones examined for BKT and LTT, as well as the sorting paralogous gene copies between species, may also bias the phylogenetic resolution. Nevertheless, the ITS1 and mtDNA data both support the monophyletic status of the yellowfin tuna group and indicate that these tropical tunas are recently derived taxa. The intraspecific variation in YFT was slightly larger than the variation observed among the tropical tuna species (see Table III) and a similar level of variation was observed even when gaps among species and repeated units were removed, contradicting the hypothesis of concerted evolutions. Although horizontal gene transfer after speciation may act to elevate the intraspecific variation, no signal for such events was shown in the mtDNA data. It appears unlikely that the mutation rate of ITS1 of YFT is significantly higher than in the other closely related tuna species. It is more likely that the observed large variation in YFT predates the speciation events and arises from maintenance of ancestral polymorphism and incomplete lineage sorting (Moore, 1995; Moran & Kornfield, 1995). Since population size and generation length may affect estimation of the molecular clock (Korey, 1981), the apparently large population size and the geographically wide and temporally extending reproductive characteristics of YFT and also SKJ (Schaefer, 2001) may serve to maintain ancestral polymorphism and newly evolved rDNA elements despite the homogenizing force of concerted evolution.

The ITS1 and mtDNA data are congruent in their separation of two large clades in the genus *Thunnus*: ALB-NBTP (plus NBTA in ITS1) in one and the remaining *Thunnus* species in the other. A hypothesis based on morphological and ecological subdivision of the genus into two subgenera (*Neothunnus* and *Thunnus*) has stood for many years (Collete, 1979), suggesting a cladistic event separated tropical (yellowfin) and temperate (bluefin) tuna lineages into these subgenera. In contrast, the molecular data suggest intermittent speciation events, in which the ancestral ALB-NBT lineage branched off first, followed by BET or SBT. The NBTA and NBTP are unique, as these two populations or sub-species

share almost identical ITS1 sequences while having distinct mtDNA. Male biased gene flow is quite unlikely, because the northern bluefin tuna do not distribute in the Indian Ocean and very few individuals have been caught in the southern hemispheres of the Atlantic and Pacific Oceans (Collette & Nauen, 1983). Since Chow & Kishino (1995) did not observe any difference between NBTA and NBTP in restriction profiles of the ITS1 fragment and allozymes, they considered that hybridization followed by mitochondrial introgression must be responsible for the sequence similarity between the NBTP and ALB mtDNAs. They could not, however, investigate further phylogenetic relationships among *Thunnus* species due to limited data from the restriction analysis on the ITS1 fragments. The present study provides sequence variation of the ITS1 locus within and between *Thunnus* tunas showing that the difference between NBTA and NBTP was comparable to, or even smaller than, variation within other species. It might be proposed that molecular drive may replace a major lineage with minor paralogous units, obscuring phylogenetic relationships. Moore (1995) indicated that the mtDNA gene tree is more likely to track the species tree than is a single nuclear gene tree. Therefore, analysis of several independent nuclear genes may be necessary to obtain an equivalent level of confidence to analyses based on mtDNA. Nevertheless, the present ITS1 data and previous allozyme data (Chow & Kishino, 1995; Pujolar *et al*., 2003) are concordant in showing very low genetic differentiation between NBTA and NBTP. Furthermore, detection of introgressive hybridization may not be difficult when investigating mtDNA haplotypes in conjunction with those of nuclear genes (Moore, 1995), as the present study illustrates. One explanation for the observed discrepancy between classifications based on the ITS1 and mtDNA in NBT is that the mitochondrial introgression occurred between the ancestral lineage of NBT which distributed or immigrated into the Atlantic, and a species in the stem gene pool which subsequently led to the SBT, BET and tropical tuna lineages.

Although the morphological and ecological similarities between NBTA and NBTP are obvious and concordant with the present ITS1 sequence data and data for several allozymes (Chow & Kishino, 1995; Pujolar *et al*., 2003), the specific status of these groups will remain unresolved until more data from the nuclear genome become available.

We wish to thank S.C. Clarke and J. Benzie for providing invaluable comments and suggestions to improve the manuscript. This work was supported in part by grants from the Japan Society for the Promotion of Science, the Ministry of Agriculture, Forestry, and Fisheries of Japan, and a Grant-in-Aid for Scientific Research on Priority Areas (B) from the Ministry of Education, Science, Sports and Culture.

References

Arnheim, N. (1983). Concerted evolution of multigene families. In: *Evolution of Genes and Proteins* (Nei, M. & Koehn, R. K., eds.), pp. 38–61. Boston, MA: Sinauer.

Alvarado-Bremer, J. R., Naseri, I. & Ely, B. (1997). Orthodox and unorthodox phylogenetic relationships among tunas revealed by the nucleotide sequence analysis of the mitochondrial DNA control region. *Journal of Fish Biology* **50,** 540–554.

Booton, G. C., Kaufman, L., Chandler, M., Oguto-Ohwayo, R., Duan, W. & Fuerst, P. A. (1999). Evolution of the ribosomal RNA internal transcribed spacer one (ITS-1) in cichlid fishes of the Lake Victoria region. *Molecular Phylogenetics and Evolution* **11,** 273–282.

Chow, S. & Inoue, S. (1993). Intra- and interspecific restriction fragment length polymorphism in mitochondrial genes of *Thunnus* tuna species. *Bulletin of National Research Institute of Far Seas Fisheries* **30,** 207–225.

Chow, S. & Kishino, H. (1995). Phylogenetic relationships between tuna species of the genus *Thunnus* (Scombridae, Teleostei): inconsistent implications from morphology, nuclear and mitochondrial genomes. *Journal of Molecular Evolution* **41,** 741–748.

Chow, S. & Ushiama, H. (1995). Global population structure of albacore (*Thunnus alalunga*) inferred by RFLP analysis of the mitochondrial ATPase gene. *Marine Biology* **123,** 39–45.

Chow, S., Okamoto, H., Miyabe, N., Hiramatsu, K. & Barut, N. (2000). Genetic divergence between Atlantic and Indo-Pacific stocks of bigeye tuna (*Thunnus obesus*) and admixture around South Africa. *Molecular Ecology* **9,** 221–227.

Chow, S., Nohara, K., Tanabe, T., Itoh, T., Tsuji, S., Nishikawa, Y., Ueyanagi, S. & Uchikawa, K. (2003). Genetic and morphological identification of larval and small juvenile tunas (Pisces, Scombridae) caught by a mid-water trawl in the western Pacific. *Bulletin of Fisheries Research Agency* **8,** 1–14.

Collette, B. B. (1979). Adaptations and systematics of the mackerels and tunas. In: *The Physiological Ecology of Tunas* (Sharp, G. D. & Dizon, A. E., eds), pp. 7–39. New York: Academic Press.

Collette, B. B. & Nauen, C. E. (1983). *Scombrids of the World*. Rome: FAO.

Collette, B. B. (1999). Mackerels, molecules, and morphology. In: *Proceedings of the 5th Indo-Pacific Fish Conference, Noumea 1997* (Seret, B. & Sire, J.-Y., eds), pp. 149–164. Paris: Société Française d'Ichtyologie.

Collette, B. B., Reeb, C. & Block, B. A. (2001). Systematics of the tunas and mackerels (Scombridae). In *Tuna Physiology, Ecology, and Evolution* (Block, B. A. & Stevens, E. D., eds), pp. 1–33. San Diego, CA: Academic Press.

Domanico, M. J., Phillips, R. B. & Oakley, T. H. (1997). Phylogenetic analysis of Pacific salmon (genus *Oncorhynchus*) using nuclear and mitochondrial DNA sequences. *Canadian Journal of Fisheries and Aquatic Sciences* **54,** 1865–1872.

Dover, G. A. (1986). Molecular drive in multigene families, how biological novelties arise, spread and are assimilated. *Trends in Genetics* **2,** 159–165.

Elliot, N. G. & Ward, R. D. (1995). Genetic relationships of eight species of Pacific tunas (Teleostei, Scombridae) inferred from allozyme analysis. *Marine and Freshwater Research* **46,** 1021–1032.

Felsenstein, J. (1993). *PHYLIP (Phylogeny Inference Package), Version 3.572*. Seattle, WA: Department of Genetics, University of Washington, Seattle, distributed by the author.

Gibbs, R. H. Jr. & Collette, B. B. (1967). Comparative anatomy and systematics of the tunas, genus *Thunnus*. *U. S. Fish and Wildlife Service Fishery Bulletin* **66,** 65–130.

Guindon, S. & Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematics Biology* **52,** 696–704.

Hasegawa, M., Kishino, H. & Yano, T. (1985). Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* **22,** 160–174.

Huyse, T., Houdt, J. V. & Volckaert, F. A. M. (2004). Paleoclimatic history and vicariant speciation in the "sand goby" group (Gobiidae, Teleostei). *Molecular Phylogenetics and Evolution* **32,** 324–336.

Korey, K. A. (1981). Species number, generation length, and the molecular clock. *Evolution* **35,** 139–147.

Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. (2001). *MEGA: Molecular Evolutionary Genetics Analysis, Version 2.01*. Tempe, AZ: Arizona State University.

Moore, W. (1995). Inferring phylogenies from mtDNA variation: Mitochondrial-gene trees versus nuclear-gene trees. *Evolution* **49,** 718–726.

Moran, P. & Kornfield, I. (1995). Were population bottlenecks associated with the radiation of the Mbuna species flock (teleostei: Cichlidae) of Lake Malawi? *Molecular Biology and Evolution* **12,** 1085–1093.

Phillips, R. B., Manley, S. A. & Daniels, T. J. (1994). Systematics of the salmonid genus *Salvelinus* inferred from ribosomal DNA sequences. *Canadian Journal of Fisheries and Aquatic Sciences* **51** (Suppl. 1), 198–204.

Pleyte, K. A., Duncan, S. D. & Phillips, R. B. (1992). Evolutionary relationships of the salmonid fish genus *Salvelinus* inferred from DNA sequences of the first internal transcribed spacer (ITS1) of ribosomal DNA. *Molecular Phylogenetics and Evolution* **1,** 223–230.

Posada, D. & Crandall, K. A. (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics* **14,** 817–818.

Pujolar, J. M., Roldán, M. I. & Pla, C. (2003). Genetic analysis of tuna populations *Thunnus thynnus thynnus* and *T. alalunga*. *Marine Biology* **143,** 613–621.

Sajdak, S. L. & Phillips, R. B. (1997). Phylogenetic relationships among *Coregonus* species inferred from the DNA sequence of the first internal spacer (ITS1) of ribosomal DNA. *Canadian Journal of Fisheries and Aquatic Sciences* **54,** 1494–1503.

Schaefer, K. R. (2001). Reproductive biology of tunas. In *Tuna Physiology, Ecology, and Evolution* (Block, B. A. & Stevens, E. D., eds), pp. 225–270. San Diego, CA: Academic Press.

Takeyama, H., Chow, S., Tsuzuki, H. & Matsunaga, T. (2001). Mitochondrial DNA sequence variation within and between *Thunnus* tuna species and its application to species identification. *Journal of Fish Biology* **58,** 1646–1657. doi: 10.1006/jfbi.2001.1575

Ward, R. D. (1995). Population genetics of tunas. *Journal of Fish Biology* **47,** 259–280.