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Intra-and interspecific restriction fragment length polymorphism in mitochondrial genes of *Thunnus* tuna species

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Abstract

Three DNA fragments of mitochondrial cytochrome *b (cytb)* and 12S rRNA *(12Sr)* genes and flanking region *(ATCO)* between ATPase6 and cytochrome oxidase subunit III genes of eight *Thunnus* tuna species were amplified by polymerase chain reaction (PCR). Restriction fragment length polymorphisms (RFLP) within and between species were investigated on these amplified fragments.

No RFLP between species was observed in *12Sr* fragment for twenty endonuclease digestions. RFLPs observed in *cytb* fragment separated tuna species into four groups. Atlantic northern bluefin *(T. thynnus thynnus)* and yellowfin *(T. albacares)* each could be descriminated from the other species. One individual out of thirtytwo Pacific northern bluefin *(T. t. orientalis)* examined had identical restriction patterns with those of the Atlantic counterpart, while the other individuals of the Pacific one were identical with albacore *(T. alalunga).* The other four species *(T. atlanticus, T. maccoyii, T. obesus* and *T. tonggol)* were found to share identical restriction profiles one another. For *ATCO* fragment, RFLPs within or between species were observed in eleven endonuclease digestions out of twenty used. Sufficient interspecific polymorphisms for separating all eight species were detected by minimum of three endonuclease digestions (*Alu* I, *Mse* I and *Hinc* II or *Hinf* I), except for one individual of the Pacific northern bluefin which was identical with the Atlantic one. Percent of shared fragments between species indicated that the Pacific northern bluefin was closer to albacore than to its Atlantic counterpart and southern bluefin which were close to yellowfin. Relatively high intraspecific polymorphisms were observed in two species (*T. albacares and T. alalunga).* These results indicated reliable use of this PCR-RFLP analysis for species identification and genetic stock structure study.

Introduction

All species of the genus *Thunnus* are very important for commercial fisheries. Considerable

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efforts on the species identification at their larval and juvenile stages have been made, because exact species identification may provide important information for studying their reproductive biology. Morphological characters have been described for identifying larvae and small juveniles of tunas (Matsumoto, 1957; Yabe *et al.*, 1966; Ueyanagi, 1966; Potthoff and Richards, 1970; Matsumoto *et al.*, 1972; Kohno *et al.*, 1982; Nishikawa, 1985; Nishikawa and Ueyanagi, 1991, 1992). Occurence and distributional pattern of melanophore and/or red pigmentation on the body and osteological characters have been the most useful characters for identifying tuna larvae. However, these characters have limited utility, where larvae of a size range from 3 to 10mm SL could be a subject for species identification (Rcihards *et al.*, 1990; Nishikawa and Ueyanagi, 1991) and red pigmentation disappeared after short period of preservation (Ueyanagi, 1966). Matsumoto *et al.* (1972) listed several useful characters for identifying small juvenile tunas. These characters also have limited size range of juveniles to be identified. Some of the characters are those of the adult, which have not yet been substantiated for juveniles (Matsumoto *et al.*, 1972). Graves *et al.* (1989) have shown that small juvenile of *T. albacares* could be electrophoretically distinguished from that of *T. obesus*, and found that some juveniles of *T. albacares* had ventral tail pigment, suggesting intraspecific variation in the pigment patterns. Further confusion in tuna species identification at their larval and/or juvenile stages could be provoked by possible geographic variation in morphological characters. In fact, Kohno *et al.* (1982) observed significant variations in the occurence of melanophores associated with larval size between Mediterranean and Pacific specimens of *T. alalunga* and *T. thynnus*. Genetic marker may substantiate these morphological characters, since DNA is virtually the same in any cell type and developmental stage of an individual. We adopted the polymerase chain reaction (PCR) method which can amplify minute amount of DNA more than 10 milion fold (Saiki *et al.*, 1988) and the restriction fragment length polymorphism (RFLP) analysis. It may be expected that PCR amplification of DNA sequence and subsequent restriction fragment length polymorphism analysis (PCR-RFLP analysis) can detect distinct differences between species.

Although allozyme markers have been employed for the genetic assessment of fish population structure, their value has been constrained because of the lack of sufficient genetic polymorphism to permit population descrimination (Billington and Hebert, 1991). Restriction fragment analysis of entire mitochondrial DNA molecule is becoming the preferred method to investigate genetic variation within and between fish species (Billington and Hebert, 1991). This conventional mtDNA method is powerful, to the extent that distinct restriction fragment patterns between three bass species could be detected in individual eggs and larvae (Graves *et al.*, 1990). However, the amount of DNA extracted from these small tissues would be insufficient for intensive genetic analysis in which use of multiple number of endonucleases is neccessary. Nucleotide sequence analysis on PCR-amplified DNA fragments was applied to some fish species to detect intra- and interspecific polymorphisms (Bartlett and Davidson, 1991; Carr and Marshall, 1991). Although the nucleotide sequence analysis has ultimate sensitivity for detecting polymorphism between sequences, it appears to consume considerable time and expense, not suited for analyzing large number of specimen. We also adopted the PCR-RFLP analysis in order to detect intraspecific genetic polymorphism. Once sufficient amount of intraspecific polymorphism using this method were detected, it would become

possible to analyze large number but very small size of specimens with much less time and expense.

In this paper, we report amplification of three mitochondrial gene regions (cytochrome *b*, 12S rRNA and flanking region between ATPase6 and cytochrome oxidase subunit III) and the results of restriction fragment length polymorphism (RFLP) analysis on these amplified DNA fragments within and between species of the genus *Thunnus*.

Materials and Methods

Tuna specimen used

Species used and source of collection are listed in Table 1. Muscle tissue (ca. 1 gr) of fresh or frozen individual was dissected at landing site or fish market, transferred on ice to the NRIFSF (National Research Institute of Far Seas Fisheries) and kept at -80°C. Specimen of Pacific northern bluefin tuna *(Thunnus thynnus orientalis)* from eastern Pacific were collected by the IATTC (Inter-American Tropical Tuna Commission), and muscle tissue (ca. 10gr) fixed in ethanol was transferred to the NRIFSF. Some specimen of longtail tuna (*T. tonggol)* were collected in Malaysia, and fixed and transferred to the NRIFSF in the same manner. Species name was abbreviated as follows in the text; albacore tuna (*Thunnus alalunga)*: ALB; bigeye tuna *(T. obesus)*: BET; blackfin tuna *(T. atlanticus)*: BKT; longtail tuna *(T. tonggol)*: LTT; Pacific northern bluefin tuna *(T. thynnus orientalis)*: PNBT; Atlantic northern bluefin tuna *(T. thynnus thynnus)*: ANBT; southern bluefin tuna *(T. maccoyii)*: SBT; and yellowfin tuna *(T. albacares)*: YFT.

Table 1. *Thunnus* tuna species used in this study.

Species	common name	abbreviation	Locality and date captured
Thunnus alalunga	albacore tuna	ALB	C. Pacific, 1991 W. Pacific, 1991
T. obesus	bigeye tuna	BET	C. Pacific, 1991 W. Pacific, 1990, 1991
T. atlanticus	blackfin tuna	BKT	Off Miami, FL, 1992
T. tonggol	longtail tuna	LTT	Gulf of Thailand, 1992 E. China Sea, 1992
T. thynnus orientalis	Pacific northern bluefin tuna	PNBT	E. Pacific, 1989 W. Pacific, 1990, 1991
T. t. thynnus	Atlantic northern bluefin tuna	ANBT	W. Atlantic, 1992
T. maccoyii	southern bluefin tuna	SBT	E. Indian, 1990 Off Tasmania, 1991 Off Cape Town, 1991
T. albacares	yellowfin tuna	YFT	E. Indian, 1990 C. Pacific, 1991 W. Pacific, 1992

DNA extraction

Larger amount of tissue (ca. 50mg) was dissected from muscle preserved in ethanol and soaked in TEK buffer (10mM EDTA, 50mM Tris, 1.5% KCl, pH 7.5) (see Chapman and Powers, 1984) at 4°C for one night prior to DNA extraction. Much smaller amount of tissue (5 to 10mg) was used for DNA extraction from frozen muscle. The muscle tissue was thinly sliced and placed in 1.5ml microcentrifuge tube containing lysis buffer. The lysis buffer consisted of 500 ul of TEK buffer, 100

ul of 10% SDS and 1 ul of 10mg/ml Proteinase K. After 30 min to 2 hrs of incubation at 60°C with occational shaking, the tube containing lysed sample was filled with phenol : chloroform (3 : 1) solution, shaken for 5 min and centrifuged at 10,000 x g for 3 min. The upper aqueous phase was transferred to 1.5ml microcentrifuge tube, and the tube was filled with chloroform : isoamyl alcohol (24 : 1) and shaken for 5 min. After centrifugation at 10,000 x g for 3 min, the upper aqueous phase was transferred to 1.5ml microcentrifuge tube, which was filled with cold absolute ethanol. Ethanol precipitation was carried out at -80°C for 10 min or at -20°C for 30 min or more, and precipitated DNA was pelletted by centrifugation at 16,000 x g for 10 min. The supernatant was decanted, and the pellet was rinsed with cold 70% ethanol and dried. The dried pellet was rehydrated in 50 ul of TE buffer (1mM EDTA, 10mM Tris-HCl, pH 8.0), and kept at -20°C.

Enzymatical amplification of three mitochondrial genes

Three sets of primers were used in this study. The two sets were abbreviated form of those described by Kocher *et al.* (1989), targeting cytochrome *b* and 12S rRNA genes, each designated *cytb* and *12Sr*. The nucleotide sequences were ; (L14838) 5'-GCTTCCATCCAACATCTCAGCATGATG-3' and (H15150) 5'-GCAGCCCCTCAGAATGATATTTGTCCTC-3' for the former and (L1091) 5'-AAACTGGGATTAGATACCCCACTAT-3' and (H1478) 5'-GAGGGTGACGGGCGGTGTGT-3' for the later. The other was designed from the consensus sequences between human (Anderson *et al.*, 1981), *Xenopus* (Roe et al., 1985) and salmon (Thomas and Beckenbach, 1989), targeting flanking region between ATPase6 and cytochrome oxidase subunit III genes, designated as *ATCO*. The nucleotide sequences were ; (L8562) 5'-CTTCGACCAATTTATGAGCCC-3' and (H9432) 5'-GCCATATCGTAGCCCTTTTTG-3'.

Gene amplification was carried out in a 0.5ml microcentrifuge tube. Components of 50 ul of reaction mixture were as follows ; 10mM Tris-HCl (pH8.3) ; 50mM KCl ; 2.0mM $MgCl_2$; 200 uM each of dATP, dCTP, dGTP and dTTP (Pharmacia Biochem.) ; 1uM each of primers ; 1.2 units of *Taq* DNA polymerase (Cetus) ; 1-2 ul of DNA template. Three drops of mineral oil were added to cover the reaction mixture. This reaction mixture was pre-heated at 93-94°C for 2 min followed by 34 cycles of amplification (92-93°C for 1 min, 52-54°C for 1 min and 70-72°C for 45 sec) with an additional cyle (92-93°C for 1 min, 52-54°C for 1 min and 70-72°C for 8 min). For amplifying *ATCO*, extention time was changed to 1.5 min.

Endonuclease digestion and agarose gel electrophoresis

The PCR products were directly subjected to restriction endonuclease digestions. Restriction endonucleases used in the present study were listed in Table 2. Except for *Acc* I, *Afl* III, *Eae* I, *Eco*NI and *Hinc* II, all recognize palindromic tetranucleotide sequence. One unit of each enzyme was applied to 1 to 3 ul of amplified DNA sample in a final volume of 5 ul of digestion mixture, which was incubated at 37°C or 55°C for 2 hrs or more. The digested samples were electrophoresed through 2.5-3% BIOGEL (BIO 101, Inc.) agarose gel in TBE buffer (90mM Tris-boric acid, 2mM EDTA). Following ethidium bromide staining, DNA bands visualized by ultraviolet (UV) illumination were photographed after 2 to 4 hrs of electrophoresis. The size of digested DNA fragments was estimated in comparison with 1-kb DNA ladder (GIBCO BRL). When a enzyme digestion generated several restriction patterns, each pattern was alphabetically labeled.

Table 2. Restriction endonucleases used in this study.

Endonucleases	recognition sequence	Manufacturer
Alu I	AG^CT	GIBCO BRL
Acc I	GT^MKAC	Boehringer Mannheim
Afl III	A^CRYGT	Boehringer Mannheim
Bsa JI	C^CNNGG	New England BioLabs
Bst UI	CG^CG	New England BioLabs
Dde I	C^TNAG	GIBCO BRL
Eae I	Y^GGCCR	New England BioLabs
Eco NI	CCTNN^NNNAGG	New England BioLabs
Fnu 4HI	GC^NGC	New England BioLabs
Hae III	GG^CC	GIBCO BRL
Hha I	GCG^C	GIBCO BRL
Hinc II	GTY^RAC	New England BioLabs
Hinf I	G^ANTC	New England BioLabs
Mbo I	G^ATC	GIBCO BRL
Mse I	T^TAA	New England BioLabs
Msp I	C^CGG	GIBCO BRL
Nla IV	GGN^NCC	New England BioLabs
Rsa I	GT^AC	GIBCO BRL
Sau 96I	G^GNCC	New England BioLabs
Scr FI	CC^NGG	New England BioLabs
Taq I	T^CAG	GIBCO BRL

K=G or T ; M=A or C ; N=A, C, G or T ; R= A or G ; Y=C or G. ^ : recognition site.

Results

Size of amplified DNA fragments

The amplified DNA fragments of *cytb*, *12Sr* and *ATCO* are shown in Fig. 1. Size of each fragments were estimated to be 355, 450 and 940 bp, respectively, and no apparent size differences between species were observed for each fragment.

Restriction endonuclease analysis for each fragment

12Sr

Twenty endonucleases were applied to this fragment amplified from two individuals of each species, and the restriction patterns were shown in Fig. 2. Eleven endonucleases (*Acc* I, *Afl* III, *Bst* UI, *Dde* I, *Eae* I, *Fnu* 4HI, *Hinc* II, *Hinf* I, *Mse* I, *Msp* I, and *Sau* 96I) appeared to have no restriction site in this fragment of all species examined. The other nine endonucleases (*Alu* I, *Bsa* JI, *Hae* III, *Hha* I, *Mbo* I, *Nla* IV, *Rsa* I, *Scr* FI and *Taq* I) had restriction sites in all species, but no polymorphism in the restricted fragment length between species was observed. It appeared, therefore, that restriction endonuclease analysis for this fragment was invalid for descriminating tuna species used

in this study, and no further effort was attempted to detect intra- and interspecific polymorphisms in this fragment.

cytb

Fifteen endonucleases were applied to this fragment. Six endonucleases (*Alu* I, *Bst* UI, *Hha* I, *Mse* I, *Nla* IV and *Scr* FI) appeared to have no restriction site in this fragment of all species, and four (*Bsa* JI, *Msp* I, *Sau* 96I and *Taq* I) had restriction sites in all species without polymorphism in the restricted fragment length between species (Fig. 3). Interspecific polymorphisms of the restricted fragment length were observed for the other five endonucleases (*Dde* I, *Eco* NI, *Hae* III, *Hinf* I and *Mbo* I) (Fig. 4), and the distributions of the digested fragments were represented by binary code (Table 3).

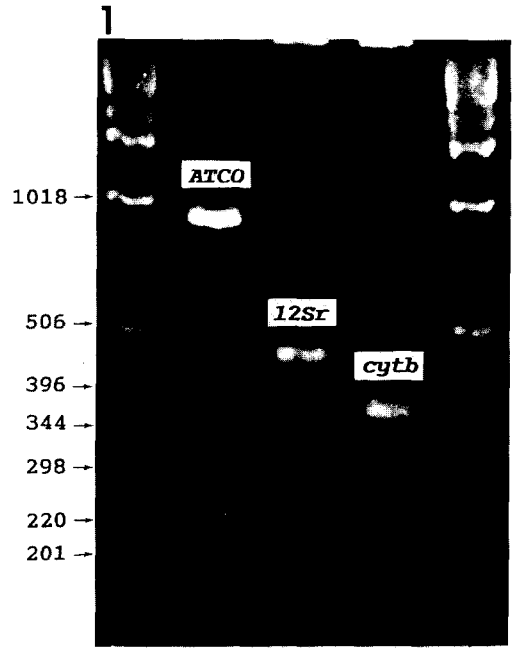


Fig. 1. Three DNA fragments amplified by polymerase chain reaction. From left to right : DNA size marker, *ATCO*, *12Sr* and *cytb* fragments, and size marker.

For *Dde* I digestion, 215 and 70bp fragments were observed (pattern B) in ANBT and one individual of PNBT (specimen No. PNBT1), while 215 and 115bp fragments (pattern A) were observed in the other species and in the other individuals of PNBT.

Eco NI had a restriction site only in YFT, generating 260 and 95bp fragments (pattern B), while no restriction site appeared in the other species (pattern A).

For *Hae* III digestion, 145 and 130bp fragments (pattern A) were observed in ANBT, PNBT1, BET, BKT, LTT, SBT and YFT, while 145 and 125bp fragments (pattern B) were observed in ALB and the other individulas of PNBT.

Hinf I digestion generated 240 and 115bp fragments (pattern A) in ANBT and PNBT1, while 200 and 115bp fragments (pattern B) were observed in the other species and in the other individuals of PNBT.

Mbo I digestion generated 295 and 60bp fragments (pattern B) in ANBT and PNBT1, while no restriction site appeared in the other species and in the other individuals of PNBT (pattern A).

Restriction patterns alphabetically designated were summarized for haplotype comparison between species (Table 4). No intraspecific polymorphism of the restricted fragment length was observed except for PNBT which comprised types 1 and 3. One individual of PNBT (PNBT1) out of thirtytwo examined represented identical restriction patterns with ANBT (type 3), where diagnostic restriction patterns were observed for *Dde* I, *Hinf* I and *Mbo* I digestions. Likewise, *Eco*

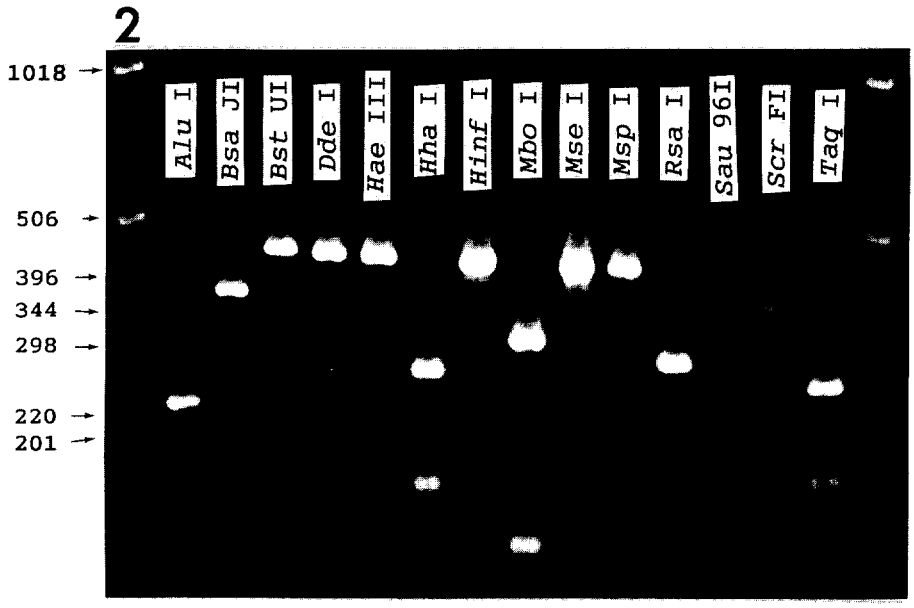


Fig. 2. Restriction profiles of *12Sr* fragment. Left and right ends are size marker. Restriction patterns of *Acc* I, *Afl* III, *Eae* I, *Fnu* 4HI, *Hinc* II and *Nla* IV having no restriction site are not shown.

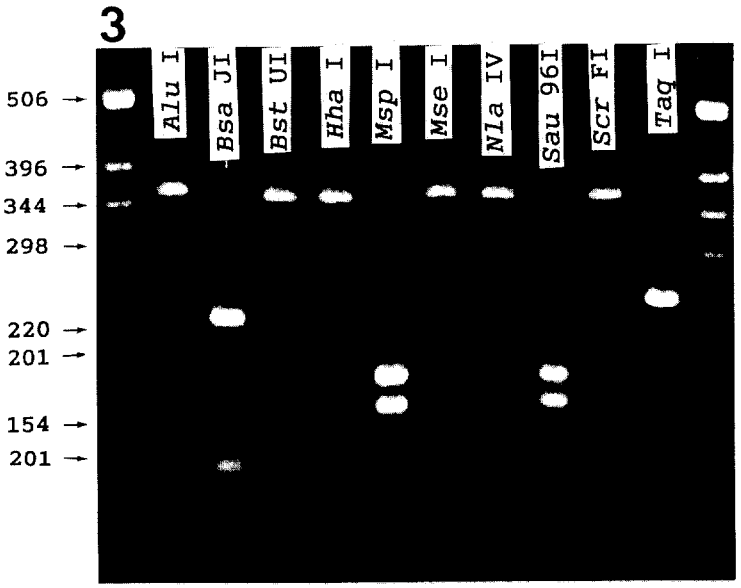


Fig. 3. Non-diagnostic restriction profiles of *cytb* fragment. Left and right ends are size marker.

Table 3. Binary representation of restricted fragment distributions of *cytb* in *Thunnus* tuna species.

Sepcies Enzymes and fragments			ALB	BET	BKT	LTT	PNBT	ANBT	SBT	YFT
Dde	I	215	1	1	1	1	1	1	1	1
		115	1	1	1	1	1	0	1	1
		70	0	0	0	0	0	1	0	0
Eco	NI	355	1	1	1	1	1	1	1	0
		260	0	0	0	0	0	0	0	1
		95	0	0	0	0	0	0	0	1
Hae	III	145	1	1	1	1	1	1	1	1
		130	0	1	1	1	0	1	1	1
		125	1	0	0	0	1	0	0	0
Hinf	I	240	0	0	0	0	0	1	0	0
		200	1	1	1	1	1	0	1	1
		115	1	1	1	1	1	1	1	1
Mbo	I	355	1	1	1	1	1	0	1	1
		295	0	0	0	0	0	1	0	0
		60	0	0	0	0	0	1	0	0
No. individuals			15	13	3	4	31	1	16	26	23

1 : fragment present ; 0 : absent.

Table 4. Composite haplotypes of tuna species in *cytb* fragment.Haplotype descriptions present five columns representing five endonucleases: 1: *Dde* I; *Eco* NI; 3: *Hae* III; 4: *Hinf* I and 5: *Mbo* I.

Type	Species		No. individual	Composite haplotype 1	2	3	4	5
1	*T. alalunga*	(ALB)	15	A	A	B	B	A
2	*T. obesus*	(BET)	13	A	A	A	B	A
2	*T. atlanticus*	(BKT)	3	A	A	A	B	A
2	*T. tonggol*	(LTT)	4	A	A	A	B	A
1	*T. thynnus*	(PNBT)	31	A	A	B	B	A
3	*orientalis*	(PNBT1)	1	B	A	A	A	B
3	*T. t. thynnus*	(ANBT)	16	B	A	A	A	B
2	*T. maccoyii*	(SBT)	26	A	A	A	B	A
4	*T. albacares*	(YFT)	23	A	B	A	B	A

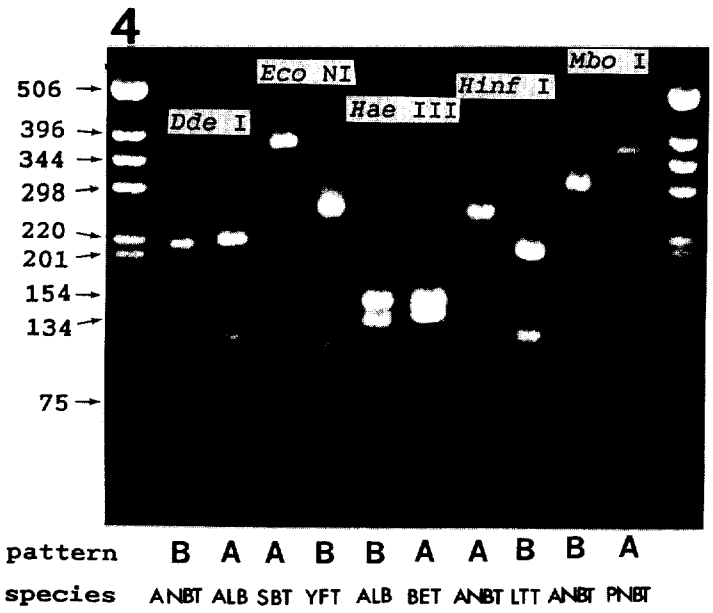


Fig. 4. Restriction profiles of *cytb* fragment showing polymorphism between species. Left and right ends are size marker.

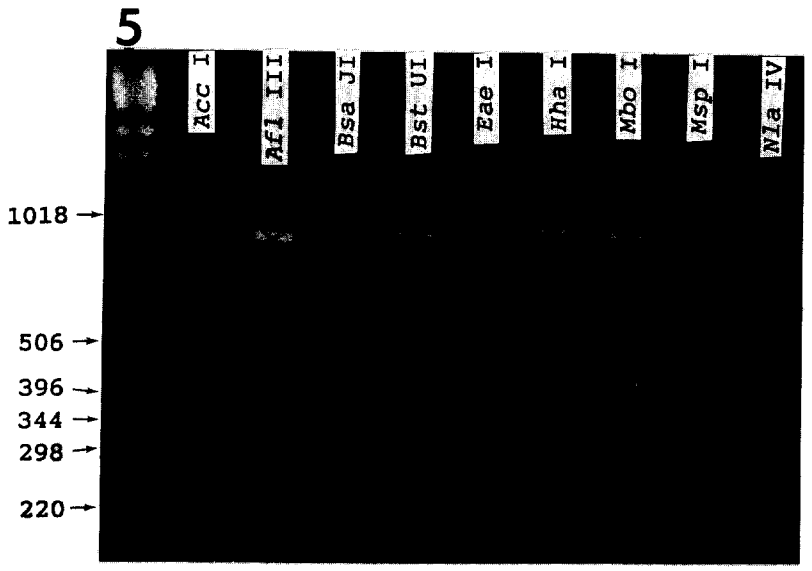


Fig. 5. Non-diagnostic restriction profiles in *ATCO* fragment. Left end is size marker.

NI digestion separated YFT (type 4) from the other species. Although the other species could be classified into two groups ; ALB and PNBT except PNBT1 (type 1) and BET, BKT, LTT and SBT (type 2), no diagnostic restriction pattern was found for species descrimination in each group.

ATCO

Twenty endonucleases were applied to this fragment. Restriction patterns by six endonucleases (*Acc* I, *Afl* III, *Bst* UI, *Hha* I, *Mbo* I and *Nla* IV) having no restriction site and by three (*Bsa* JI, *Eae* I and *Msp* I) having restriction sites but showing no length variation on the restricted fragments between species were shown in Fig. 5.

The other eleven endonucleases were found to give intra and/or interspecific polymorphisms, and the restriction patterns by each enzyme were shown in Figs. 6 to 16. The distributions of the digested fragments were represented by binary code (Table 5).

Five restriction patterns were observed for *Alu* I digestion (Fig. 6), where 420, 295, 80 and 70bp fragments (pattern C) were observed in PNBT except PNBT1, 420, 295, 120 and 70bp (pattern B) were in ALB, 420, 295 and 150bp (pattern A) were in BET, BKT and one individual of YFT, 295, 280, 190 and 150bp (pattern D) were in ANBT and PNBT1, and 295, 280 and 150bp (pattern E) were in LTT, SBT and the other individuals of YFT.

Dde I digestion represented three patterns (Fig. 7), where 380, 280 and 195bp fragments (pattern A) were observed in ALB, BET, BKT and PNBT (except PNBT1), 280, 210, 195 and 170bp fragments (pattern C) were in LTT, ANBT, PNBT1, and one individual of SBT, and 360, 280 and 210bp fragments (pattern B) were in the other individulas of SBT. All three patterns were observed in YFT at different frequency.

LTT was distinct from other species in *Fnu* 4HI digestion, where 480, 250 and 180bp fragments (pattern B) were observed. The other species had 510bp fragment instead of 480bp fragment (pattern A) (Fig. 8).

In *Hae* III digestion, 310, 180, 160, 140 and 80bp fragments (pattern A) were observed in all species except for ANBT and PNBT1 which had no 140bp fragment (pattern B) (Fig. 9).

Hinc II digestion indicated distinct fragment pattern A (720 and 220bp) in BKT and LTT from the other species which possessed 470, 250 and 220bp fragments (pattern B) (Fig. 10).

In *Hinf* I digestion, 690, 135 and 110bp fragments (pattern A) were observed in ALB, BET, PNBT (except PNBT1), SBT and YFT, while no 135bp fragment was observed in BKT, LTT, ANBT and PNBT1 (pattern B) (Fig. 11).

Mse I digestion revealed high polymorphism within and between species, and eight restriction patterns were observed (Fig. 12). Nine individuals of ALB and all but one (PNBT1) of PNBT were identical in the restriction pattern (260, 195 and 100bp fragments) (pattern G). In the other individuals of ALB, 260, 250, 195 and 100bp fragments (pattern F) were observed in eleven individuals, while 250, 195, 135 and 100bp fragments (pattern H) were observed in one. BET was distinct from all of the other species, where 300, 230 and 195bp fragments (pattern A) were observed. ANBT, PNBT1 and fourtythree individuals of SBT possessed identical restriction pattern, where 270, 230, 195 and 100bp fragments (pattern E) were observed. The other individual of SBT shared the same restriction

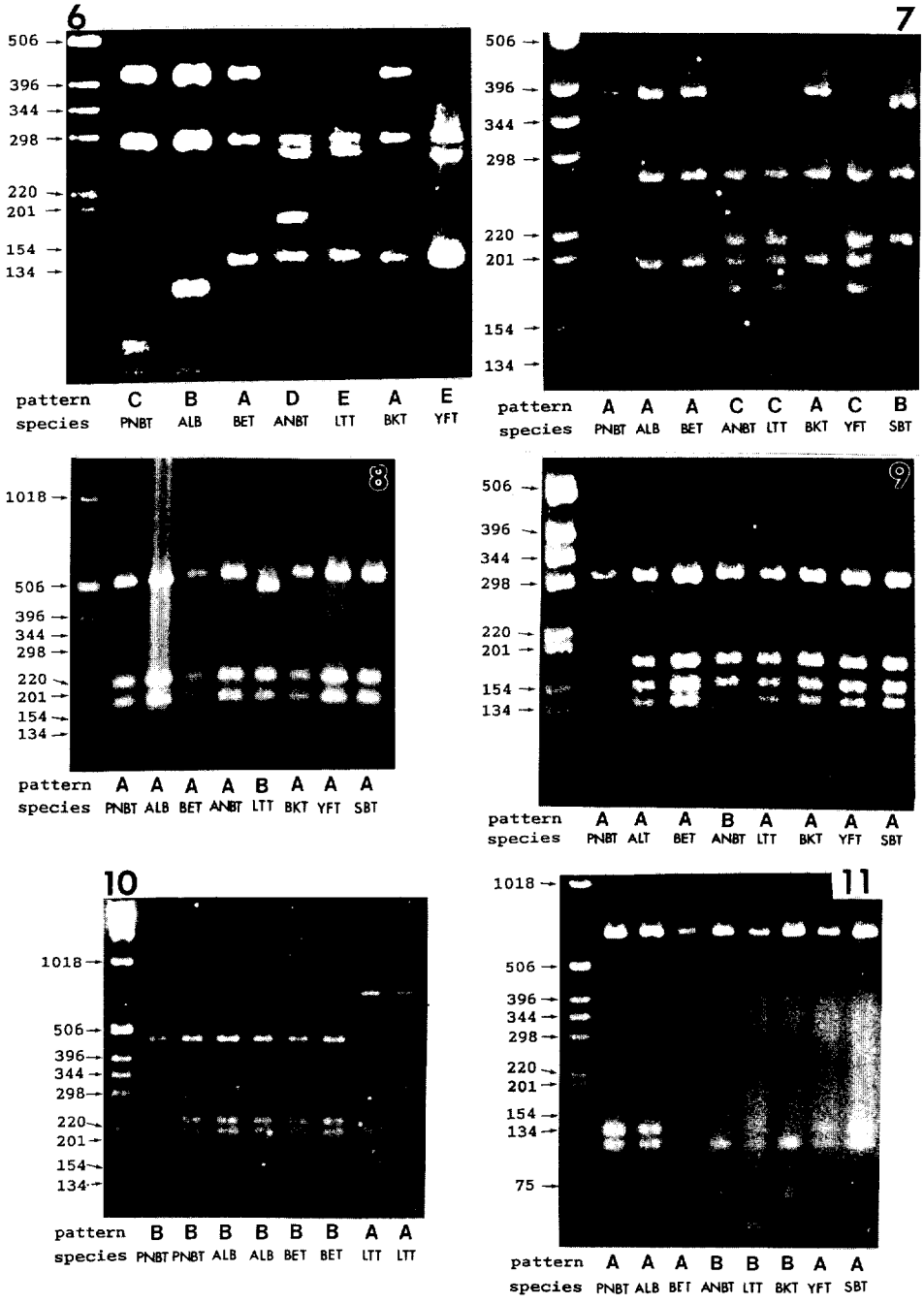
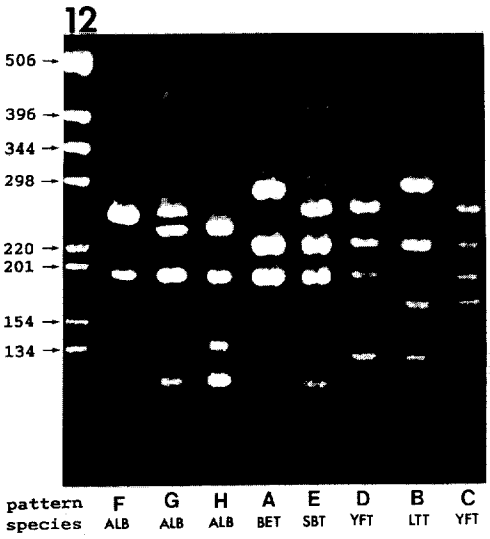


Fig. 6-16. Restricon profiles of ATCO fragment patterns showing intra-and interspeific polymorphisms. Left end is size marker. (6)*Alu* I,(7)*Dde* I,(8)*Fnu* 4HI,(9) *Hae* III, (10) *Hinc* II, (11) *Hinf* I, (12) *Mse* I, (13) *Rsa* I, (14) *Sau* 96I, (15) *Scr* FI and (16) *Taq* I.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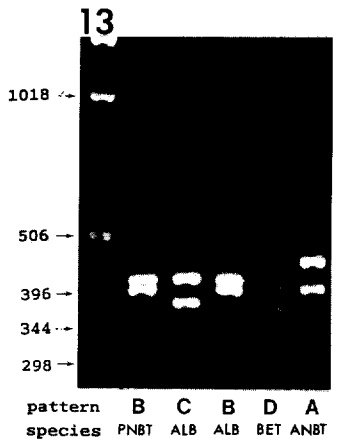
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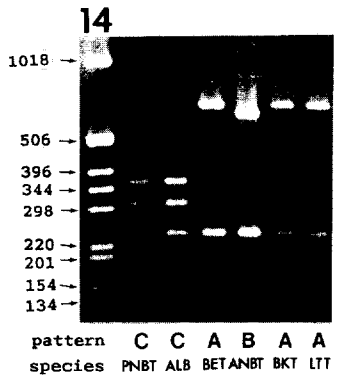
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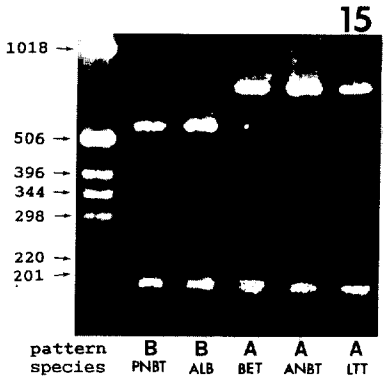
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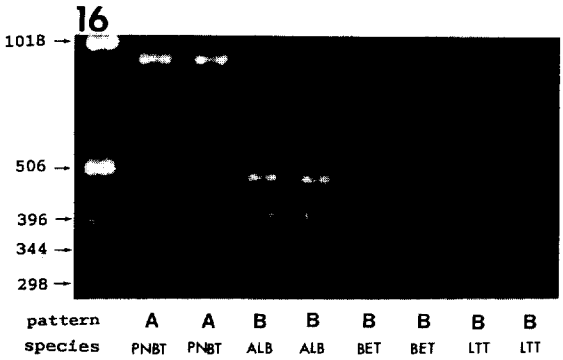
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pattern A A B B B B B B
species PNBT PNBT ALB ALB BET BET LTT LTT

Table 5. Binary representation of restricted fragment distributions of *ATCO* in *Thunnus* tuna species.

Species Enzymes and fragments		ALB				BET	BKT	LTT	PNBT		ANBT	SBT			YFT			
Alu I	420	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	1
	295	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	285	0	0	0	0	0	0	1	0	1	1	1	1	1	1	1	1	0
	190	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0
	150	0	0	0	0	1	1	1	0	1	1	1	1	1	1	1	1	1
	120	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
	80	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
	70	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0
Dde I	380	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	1
	360	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0
	280	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	210	0	0	0	0	0	0	1	0	1	1	1	1	1	1	1	1	0
	195	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0	1	1
	170	0	0	0	0	0	0	1	0	1	1	0	0	1	1	0	1	0
Fnu 4HI	510	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1
	480	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
	250	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	180	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hae III	310	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	180	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	160	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	140	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1
	80	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinc II	720	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
	470	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1
	250	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1
	220	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hinf I	690	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	135	1	1	1	1	1	0	0	1	0	0	1	1	1	1	1	1	1
	110	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Mse I	300	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0
	270	0	0	0	0	0	1	0	0	1	1	1	1	0	1	1	1	1
	260	1	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
	250	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
	230	0	0	0	0	1	1	1	0	1	1	1	1	1	1	1	1	1
	195	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1
	175	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0
	135	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
	120	0	0	0	0	0	1	1	0	0	0	0	0	1	1	1	0	1
	100	1	1	1	1	0	0	0	1	1	1	1	1	0	0	0	0	0
Rsa I	450	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0
	420	1	1	1	1	0	1	1	1	0	0	1	0	1	1	1	1	1
	405	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	380	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	370	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
Sau 96I	670	0	0	0	0	1	1	1	0	0	0	1	1	1	1	1	1	1
	640	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0
	360	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0
	310	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0
	270	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Scr FI	755	0	0	0	0	1	1	1	0	1	1	1	1	1	1	1	1	1
	580	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0
	185	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Taq I	940	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
	520	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1
	420	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1
No. individuals		11	6	3	1	16	3	6	17	1	15	41	2	1	22	3	2	1

1 : fragment present : 0 : absent.

Table 6. Composite haplotypes of tuna species in *ATCO* fragment. Haplotype descriptions present 11 columns representing 11 endonucleases: 1: *Alu* I; 2: *Dde* I; 3: *Fnu* 4HI; 4: *Hae* III; 5: *Hinc* II; 6: *Hinf* I; 7: *Mse* I; 8: *Rsa* I; 9: *Sau* 96I; 10: *Scr* FI; and 11: *Taq* I.

Type	Species		NO. individual	Composite haplotype										
				1	2	3	4	5	6	7	8	9	10	11
1	*T. alalunga*	(ALB)	11	B	A	A	A	B	A	F	B	C	B	B
2			6	B	A	A	A	B	A	G	B	C	B	B
3			3	B	A	A	A	B	A	G	C	C	B	B
4			1	B	A	A	A	B	A	H	B	C	B	B
5	*T. obesus*	(BET)	16	A	A	A	A	B	A	A	D	A	A	B
6	*T. atlanticus*	(BKT)	3	A	A	A	A	A	B	D	B	A	A	B
7	*T. tonggol*	(LTT)	6	E	C	B	A	A	B	B	B	A	A	B
8	*T. thynnus*	(PNBT)	17	C	A	A	A	B	A	G	B	C	B	A
9	*orientalis*	(PNBT1)	1	D	C	A	B	B	B	E	A	B	A	B
9	*T. t. thynnus*	(ANBT)	15	D	C	A	B	B	B	E	A	B	A	B
10	*T. maccoyii*	(SBT)	41	E	B	A	A	B	A	E	B	A	A	B
11			2	E	B	A	A	B	A	E	D	A	A	B
12			1	E	C	A	A	B	A	B	B	A	A	B
13	*T. albacares*	(YFT)	22	E	C	A	A	B	A	D	B	A	A	B
14			3	E	B	A	A	B	A	D	B	A	A	B
15			2	E	C	A	A	B	A	C	B	A	A	B
16			1	A	A	A	A	B	A	D	B	A	A	B

pattern B (300, 230, 175 and 120bp fragments) with LTT. Twentysix individuals of YFT shared the same pattern D (270, 230, 195 and 120bp fragments) with BKT, while two individuals of YFT had a distinct pattern C (270, 230, 195 and 175bp fragments).

Rsa I digestion represented four restriction patterns (Fig. 13). BKT, LTT, PNBT (except PNBT1), YFT, eighteen individuals of ALB and fourtytwo of SBT were observed to have the same fragment pattern B (420 and 405bp). ANBT and one individual of PNBT (PNBT1) had 450 bp fragment instead of 420bp fragment (pattern A). Three individuals of ALB also had distinct pattern C (420 and 380bp fragments). Three individuals of SBT were found to share the same pattern D (405 and 370bp) with BET.

Sau 96I digestion revealed distinct patterns in ALB, PNBT and ANBT from the other species (Fig. 14). ANBT and one individual of PNBT (PNBT1) had 640 and 270bp fragments (pattern B), while ALB and the other individuals of PNBT had 360, 310 and 270bp fragments (pattern C). The other species had 670 and 270bp fragments (pattern A).

Scr FI digestion generated 580 and 185bp fragments in ALB and PNBT (except PNBT1) (pattern B), while 755 and 185bp fragments were observed in the other species (pattern A) (Fig. 15).

Taq I appeared to have no restriction site in all but one of PNBT (pattern A), while 520 and 420bp fragments (pattern B) were generated in the other species and one individual of PNBT (PNBT1) (Fig. 16).

Restriction patterns alphabetically designated were summarized for haplotype comparison between species (Table 6). Except for ANBT and one individual of PNBT (PNBT1) which shared identical restriction patterns in all endonucleases examined, all species could be descriminated one another by using minimum of three endonucleases (*Alu* I, *Mse* I and *Hinc* II or *Hinf* I). Relatively

high intraspecific polymorphism was observed in ALB and YFT, in each of which four haplotypes were found (types 1 to 4 and 13 to 16, respectively). On the other hand, polymorphism was much lower in PNBT (types 8 and 9) and SBT (types 10 to 12), and nil in BET (type 5), BKT (type 6), LTT (type 7) and ANBT (type 9).

Discussion

Although allozyme electrophoresis techniques have been recommended for identifying fish species (Smith and Crossland, 1977 ; Dotson and Graves, 1984 ; Graves *et al.*, 1989), these have limitted utility for fish embryo and larvae which would not offer good quality and amount of proteins. Further, enzyme expression may change ontogenetically and/or vary from tissue to tissue. The polymerase chain reaction (PCR) may overcome these problems. Bartlett and Davidson (1991) analysed DNA sequences of cytochrome *b* gene amplified by the PCR and found nucleotide substitutions within and between four species of the genus *Thunnus*. The present study indicated that RFLP analysis on PCR-amplified gene fragment is much simpler and less time consuming and that RFLP analysis on *cytb* fragment alone could separate the four species of tunas (ALB, BET, ANBT and YFT) examined by Bartlett and Davidson (1991). However, RFLP analysis on *cytb* and *12Sr* fragments failed to descriminate all eight tuna species. Higher polymorphisms which made complete descrimination between *Thunnus* tuna species possible were obtained in *ATCO* fragment. Thus, probability to find polymorphism in restriction fragment length must become higher in longer fragment, since the number of restriction site is subjected to the length of fragment analyzed. Differentiation between species may also vary among gene regions in mitochondrial DNA molecule, because higher polymorphisms were observed in *cytb* fragment than in *12Sr*. Kawakawa (*Euthynnus affinis*) and skipjack (*Katsuwonus pelamis*) also represented identical restriction patterns on *12Sr* fragment (unpublished data) with those of tunas used in this study, indicating a quite conserved nature in this gene region. In contrast, intra- and interspecific polymorphism on *12Sr* fragment were found in the western Atlantic snapper species (Chow *et al.*, 1993). Moreover, PCR-RFLP analysis on *cytb* fragment could separate six billfish species of the Pacific and five of the Atlantic (Chow, 1992). These indicate that differentiation of a given gene between closely related species may vary among taxa. Thus, examination and selection of target gene region and length of fragment amplified would be critical for the PCR-RFLP analysis on investigating polymorphism in a given taxon. Embryos, larvae and small juveniles of tunas to which morphological species identification have been ambiguous or impossible, may be analyzed by the method demonstrated in this study. Actually, mitochondrial DNA extracted from ethanol-preserved embryo and larvae of snapper species (the subfamily Lutjaninae) was successfully amplified and subjected to the RFLP analysis (Chow *et al.*, 1993).

It was demonstrated that in some species relatively high intraspecific polymorphisms could be detectd by the PCR-RFLP analysis. This method, however, appears to miss many nucleotide substitutions, since Bartlett and Davidson (1991) using nucleotide sequence analysis detected considerably high polymorphism in bigeye tuna (*T. obesus*) but no polymorphism was detected in the

present study. In order to detect higher intraspecific polymorphisms which may be useful for genetic stock structure study, amplification of longer fragment and/or searching other gene regions accumlating higher poymorphism may be neccessary for some species. It is now possible to compare genetic population structure of embryo or larvae collected in spawning ground and that of adult or juvenile captured at high sea, which may provide important information for studying stock structure of highly migrating fish species such as tunas.

Using five informative restriction profiles in *cytb* and eleven in *ATCO*, percent of shared fragments between specimens was calculated. Highest value (93%) was observed between majority of albacore and the Pacific northern bluefin tunas, followed by that (90%) between majority of southern bluefin and yellowfin tunas. Percent of shared fragments between the Atlantic northern bluefin and majority of the Pacific northern bluefin tunas was lowest (60%), while the Atlantic northern bluefin tuna was found to share more fragments with southern bluefin and yellowfin tunas (c.a. 80%). However, occurrence of an Atlantic type of individual (PNBT1) in the Pacific specimen of northern bluefin tuna indicates large but incomplete genetic differentiation between Atlantic and Pacific northern bluefin tunas. Nucleotide sequence analysis on *cytb* fragment of PNBT1 (unpublished data) also indicated that this individual had identical nucleotide sequence with that of the Atlantic counterpart reported by Bartlett and Davidson (1991). These results do not agree with the phylogenetic relationships among *Thunnus* tuna species proposed by Iwai *et al.* (1965) using morphological characters and by Sharp and Pirages (1978) using allozyme analysis, in which these three types of bluefin tunas were closely categorized. To elucidate their phylogenetical relationships, analysis on the nuclear genes must be neccessary.

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マグロ属（*Thunnus*）種内および種間における mtDNA 制限酵素切断型多型

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摘　要

マグロ類 mtDNA 分子内の 3 種類の遺伝子領域（cytochrome *b*, 12S rRNA, ATPase）から PCR 法によって 3 種類の DNA 断片（*cytb, 12Sr, ATCO*）を増幅し、各断片について制限酵素処理を行なった。マグロ属の 8 種間および種内での切断型多型を検索し種判別、系群構造の遺伝学的分析に対する本法の有効性について検討した。増幅した断片の長さはそれぞれ355, 450, 940塩基対であり種間における長さの差は見られなかった。*12Sr* 断片では、20種類の制限酵素による切断型に魚種間で差が見られなかった。*cytb* 断片では、大西洋クロマグロ（*T. thynnus thynnus*）とキハダ（*T. albacares*）においてそれぞれ特異的切断型が見られ他種から分けられた。太平洋クロマグロ（*T. t. orientalis*）32個体中 1 個体が大西洋クロマグロと同一の切断型を示し、他個体はビンナガ（*T. alalunga*）と同一であった。タイセイヨウマグロ（*T. atlanticus*）、ミナミマグロ（*T. maccoyii*）、メバチ（*T. obesus*）、コシナガ（*T. tonggol*）4 種間の切断型に差は見られなかった。*ATCO* 断片では使用した20種類の制限酵素のうち11種で種間および種内個体間での切断型多型が見られた。上記の太平洋クロマグロ 1 個体はこの断片においても大西洋クロマグロと同一の切断型を示した。この 1 個体を除けば、3 種類の制限酵素（*Alu*I, *Mse*I, *Hinc*II または *Hinf* I）による切断型を用いて全種の判別が可能であった。切断型を比較した場合、太平洋のクロマグロは大西洋型のものよりむしろヒンナガに似ていること、ミナミマグロはクロマグロよりもキハダに似ていることが示され、従来の系統関係を再検討する必要があるものと考えられた。ビンナガ、キハダではそれぞれ 4 種類の切断型が見られ、短い DNA 断片でも比較的高いレベルの種内切断型多型の検出が可能であることが示唆された。本法はアルコール保存された微小な卵稚仔標本も分析の対象とすることができるため、特に高度回遊性魚種における系群構造の遺伝学的分析に有効であるものと考えられる。