IDENTIFICATION OF BILLFISH SPECIES USING MITOCHONDRIAL CYTOCHROME B GENE FRAGMENT AMPLIFIED BY POLYMERASE CHAIN REACTION

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SUMMARY

Mitochondrial cytochrome b gene of ten billfish species (Istiophorus albicans, I. platypterus, Makaira indica, M. mazara, M. nigricans, Tetrapturus albidus, T. angustirostris, T. audax, T. pfluegeri and Xiphias gradius) collected from the Atlantic, Indian and Pacific Oceans were amplified by polymerase chain reaction (PCR). The amplified DNA fragments were 350bp and no apparent size difference was observed among species. Thirteen restriction endonucleases were applied to the amplified fragments in order to investigate restriction fragment length polymorphism (RFLP) within and among species. RFLP among species was observed in digestions by nine endonucleases. RFLP within species was observed in the Atlantic sailfish (I. albicans) and swordfish (X. gladius). Identical restriction profiles were observed between the Atlantic and Pacific blue marlin (M. mazara and M. nigricans) and between the Atlantic white marlin (T. albidus) and striped marlin (T. audax). Simple identification procedures were designed for each oceanic sample, where all species in the Indo-Pacific and five species in the Atlantic could be distinguished by the genotype analysis of three endonuclease digestions (Alu I, Bsa JI and Taq I) and of two (Bsa JI and Taq I), respectively.

RESUME

Les gènes du cytochrome b de dix espèces d'istiophoridés (Istiophorus albicans, I. platypterus, Makaira indica, M. mazara, M. nigricans, Tetrapturus albidus, T. angustirostris, T. audax, T. pfluegeri et Xiphias gladius) prélevées dans les océans Atlantique, Indien et Pacifique ont été amplifiés au moyen d'une réaction en chaîne de polymérase. Les fragments amplifiés de DNA étaient de 360 bp, et aucune différence apparente de grandeur n'a été observée entre les espèces. Treize endonucléases de restriction ont été appliquées aux fragments amplifiés pour rechercher le polymorphisme de la longueur des fragments de restriction (RFLP) au sein des espèces et entre elles. Un RFLP inter-spécifique a été observé chez le voilier de l'Atlantique (I. albicans) et l'espadon (X. gladius). Des profils de restriction identiques ont été observés entre le makaire bleu de l'Atlantique et celui du Pacifique (M. mazara et M. nigricans), et entre le makaire blanc de l'Atlantique (T. albidus) et le Tetrapturus audax. Un processus simple d'identification a été élaboré pour chaque échantillon océanique, permettant de distinguer respectivement toutes les espèces indo-pacifiques et cinq espèces atlantiques au moyen de l'analyse du génotype de trois (AluI, BsaJI et TaqI) et de deux (BsaJI et TaqI) digestions d'endonucléases, respectivement.

RESUMEN

Los genes mitrocondriales que codifican el citocromo b de diez especies de marlín (Istiophorus albicans, I. platypterus, Makaira indica, M. mazara, M. nigricans, Tetrapturus albidus, T. angustirostris, T. audax, T.pfluegeri y Xiphias gladius) procedentes de los océanos Atlántico, Indico y Pacífico, fueron amplificados mediante la reacción en cadena de la polimerasa (PCR). Los fragmentos de ADN amplificados tenían 360 pares de bases y no se observó diferencia de tamaño entre las especies. Se emplearon trece endonucleasas de restricción sobre los fragmentos amplificados, con el fin de determinar el polimorfismo

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de los subfragmentos de restricción (RFLP) dentro de cada especie y entre las distintas especies. El polimorfismo entre las distintas especies se observó después de digerir los fragmentos con nueve endonucleasas. El polimorfismo dentro de cada especie se determinó para el pez vela atlántico (I. albicans) y el pez espada (X. gladius). Se obtuvieron patrones de restricción idénticos en la aguja azul del Atlántico y el Pacífico (M. mazara y M. nigricans) por una lado, y en la aguja blanca del Atlántico (T. albidus) y el pez aguja (T. audax), por otro. Se establecieron protocolos simples de identificación para cada muestra oceánica. Así, todas las especies del Indo-Pacífico pudieron distinguirse por análisis genotípico con tres enzimas de restricción (AluI, BsaJI y TaqI) y cinco especies procedentes del Atlántico se identificaron por medio de dos de estas enzimas (BsaJI y TaqI).

1. INTRODUCTION

It has been recognized that the analyses of mtDNA may yield important data for understanding genetic constitution of fish stocks (Ferris and Berg, 1986) because of the rapid evolutionary rate (Brown *et al.*, 1979) and the strict maternal inheritance (Hutchinson *et al.*, 1974). Analysis on the restriction fragment length polymorphism (RFLP) has now been widely adopted for quantifying genetic differences among populations. However, procedures for purifying mtDNA or detecting mtDNA from crude DNA sample with endo-labelled probe by Southern blotting are tedious and not suited for rapid analysis of large numbers of samples. Further, these techniques virtually have little power for intensive DNA analysis on minute amount of samples such as fish embryo or larvae. The polymarase chain reaction (PCR) which can enzymatically amplify selected DNA sequences (Saiki *et al.*, 1988) may resolve these problems. For example, direct nucleotide sequence of mitochondrial cytochrome *b* gene fragments amplified by PCR has been applied to detect sequence variations among four species of tuna (Bartlett and Davidson, 1991) and that within Atlantic cod (Carr and Marshall, 1991). In order to detect genetic differences between species, however, it might not be neccessary to use nucleotide sequence analysis which still consumes time and expense.

In this study, I adopt PCR to amplify cytochrome *b* gene of ten billfish species and report RFLP of the amplified fragments within and among species.

2. MATERIALS AND METHODS

2.1 Sample collection

Species used in this study, number of specimen and source of collection are listed in Table 1. All fish specimen were caught during 1991 and 1992.

Most of the Indo-Pacific billfish species were collected by the NRIFSF (National Research Institute of Far Seas Fisheries) staffs in Yaizu, one of the largest landing site in Japan. Muscle tissues (< 1gr) of each individual were dissected at the landing site, transferred to the NRIFSF and kept at -80° C. All species collected at Yaizu were from the western Pacific except for five individuals of shortbill spearfish (*Tetrapturus angustirostris*) which were caught in the central Pacific. One individual of Indo-Pacific sailfish (*Istiophorus platypterus*) was caught by research cruise in the Indian Ocean. Swordfish specimen from the Indian Ocean were collected by Mr. M. Yesaki, IPTP (Indo Pacific Tuna Development and Managemnt Programme), and the frozen muscle tissues (< 1gr) were transferred to the NRIFSF.

Muscle tissues of all of the Atlantic billfish species used were given by courtesy of Dr. J. X. Hartmann, FAU (Florida Atlantic University) and of the NMFS (National Marine Fisheries Service) at Miami. Extraction of total DNA from the muscle tissue was carried out in the Division of Marine Biology and Fisheries, University of Miami, and the dried DNA pellet samples were transferred to the NRIFSF for subsequent analyses.

2.2 DNA extraction

Total genomic DNA was extracted from frozen muscle. Minced muscle tissue (c.a. 50 mg) was placed in 1.5 ml microcentrifuge tube containing 0.5ml of TEK buffer (50mM Tris, 10mM EDTA, 1.5% KCl,

pH7.5)(see Chapman and Powers, 1984). Sodium dodecyl sulphate (SDS) and proteinase K were added to 1% and 6ug/ml, respectively. After 1 to 2 hrs of incubation at 65° C with occasional shaking, the sample was mixed with 1ml of phenol:chloroform (3:1) solution, shaken for 5 min and centrifuged at 8,000 x g for 5 min. The upper aqueous phase was recovered and extracted with 1ml chloroform:isoamyl alcohol (24:1) solution to remove traces of phenol. The upper aqueous phase was recovered and mixed with 2-2.5 volume of cold absolute ethanol. The sample was kept at -20° C for 1 hr or more for DNA precipitation. DNA was pelleted by centrifugation at 12,000 x g for 10 min. The supernatant was decanted, and the pellet was rinsed with 70% ethanol and dried. The dried pellet was rehydrated in 100ul of TE buffer (1mM EDTA, 10mM Tris-HCl, pH8.0), and kept at -20° C.

2.3 Amplification of mitochondrial cytochrome b *gene*

Two primers targetting mitochondrial cytochrome *b* gene were abbreviated forms of those described by Kocher *et al.* (1989). The nucleotide sequences of the primers were; (L14838) 5'GCTTCCATCCAACA-TCTCAGCATGATG-3' and (H15150) 5'-GCAGCCCCTCAGAATGATATTTGTCCTC-3'. Polymerase chain reaction (PCR) was carried out in a 0.5 ml microcentrifuge tube. Components of 50ul of reaction mixture were as follows; 10mM Tris-HCl (pH8.3); 50mM KCl; 2.0mM $MgCl_2$; 250uM each of dATP, dCTP, dGTP, and dTTP; 1uM each of primers; 1.2 units of *Taq* DNA polymerase; and 1 to 2 ul of DNA template. Two 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 30 cycles of amplification (90-93° C for 1 min, 50-52° C for 1 min and 71-72° C for 1 min) with an additional cycle (90-93° C for 1 min, 50-52° C for 1 min and 70-72° C for 5 min). After the amplification, 0.3ml of sterilized water was added, followed by chloroform extraction and ethanol precipitation. After centrifugation at 16,000 x g for 15 min, the pellet was rinsed with 70% ethanol, dried and rehydrated with 20 ul of TE buffer.

2.4 Endonuclease digestion and agarose gel electrophoresis

Thirteen restriction endonucleases which recognize palindromic tetra nucleotide sequence were used in this study (Table 2). One unit of each enzyme was applied to 1 ul of amplified DNA sample in a final volume of 5 ul of digestion mixture. The digested samples were electrophoresed through 3% BIOGEL (BIO 101, Inc.) agarose gel in TBE buffer (90mM Tris-boric acid; 2mM EDTA). 2ul of 10mg/ml ethidium bromide was added to the electrophoresis buffer. DNA bands were photographed after 1.5 to 3 hrs of electrophoresis.

3. RESULTS

The primers used in this study successfully amplified 350 bp DNA fragment of cytochrome *b* gene in all species without apparent size differences among species. The representative restriction patterns of this 350bp fragment by each restriction endonuclease are shown in Fig. 1. The size of each restricted fragment was estimated in comparison with size standards (1kb ladder, BRL) and shown in Table 3. Different restriction profiles were designated A, B, C or D types in each endonuclease digestion. For genotype analysis, composite digestion patterns of each species are shown in Table 4.

Two endonucleases (*Mbo* I and *Rsa* I) appeared to have no restriction site in the 350bp fragments of all species. Each of *Hha* I and *Hin* fI had one restriction site showing no restriction fragment length polymorphism (RFLP) among species.

RFLP among species was observed in the digestions by nine endonucleases (*Alu* I, *Bsa* JI, *Bst* UI, *Dde* I, *Hae* III, *Msp* I, *Sau* 96I, *Scr* FI and *Taq* I). No restriction site for *Alu* I was appeared in Atlantic (ABM) and Pacific (PBM) blue marlin, and longbill (LBS) and shortbill (SBS) spearfish (Fig. 1A, lanes 5 and 6). In contrast, *Alu* I digestion produced 315bp fragment in swordfish (SWF) and 310bp fragment in Atlantic sailfish (ASA), Atlantic white marlin (AWM), black marlin (BLM), Pacific sailfish (PSA) and striped marlin (PSM). *Bsa* JI had no restriction site in Atlantic and Pacific blue marlin, black marlin and longbill and shortbill spearfish, while it cleaved the 350bp fragment into 265 and 85bp fragments in Atlantic white marlin and striped marlin, 230 and 120bp fragments in Atlantic and Pacific sailfish, and 195 and 120bp fragments in swordfish. *Bst* UI cleaved the 350bp fragment into 250 and 100bp fragments only in swordfish. *Dde* I had restriction sites for all species, producing 325 bp fragment in swordfish, 295bp

fragment in Atantic white marlin and striped marlin, and 230bp fragment in the other species. *Hae* III digestion indicated intraspecific RFLP in swordfish. Among twenty five swordfish individuals examined, two (one from Pacific and the other from Indian Ocean) represented a different restriction profile (140 and 130bp fragments) compared with that of the other individuals (130 and 100bp fragments). *HaeIII* also cleaved the 350bp fragments of all other species into 155 and 120bp fragments without RFLP among species. *Msp* I had a restriction site producing 210 and 140 bp fragments in Atlantic white marlin, Atlantic sailfish, black marlin, longbill and shortbill spearfish and striped marlin, while no restriction site was observed in the other species. *Sau* 96I had a restriction site only in shortbill spearfish producing 240 and 110bp fragments. *Scr* FI had no restriction site in swordfish, Atlantic and Pacific blue marlin, black marlin and one individual of Atlantic sailfish out of fifteen examined, while this endonuclease digestion produced 220 and 120bp fragments in the other species. *Taq* I digestion indicated absence of restriction site in swordfish, while produced 185 and 165 bp fragments in shortbill spearfish and Atlantic and Pacific sailfish, and 165 and 130bp fragments in the other species.

Since identical restriction profiles were observed between Atlantic and Pacific blue marlin and between Atlantic white marlin and the striped marlin, complete descrimination between billfish species was not accomplished in this study (Table 4). However, separating the specimens into two groups (Atlantic and Pacific) could make it possible to identify all species used in this study. Among nine endonucleases representing RFLP between species, *Ahu* I, *Bsa* JI and *Taq* I which generated larger number of different restriction profiles were chosen. Simple identification procedures for each oceanic specimen using these endonucleases were designed and shown in Fig. 2. For the Pacific species, *Bsa* JI digestion could identify sailfish, striped marlin and swordfish. Among three species remained, shortbill spearfish could be descriminated from the other two (black marlin and blue marlin) by *Taq* I digestion. The black marlin and blue marlin could be separated by *Ahu* I digestion. *Ahu* I can be replaced by *Msp* I. For the five Atlantic species used in this study, Atlantic white marlin, sailfish and swordfish could be identified by *Bsa* JI digestion, and *Taq* I could descriminate other two species (blue marlin and longbill spearfish).

4. DISCUSSION

RFLP analysis on PCR products demonstrated in this study could represent distinct genetic differences among billfish species. Though subjected to the level of RFLP within the species, once species-specific restriction profiles were detected, the analytical procedure using only a limited number of diagnostic endonucleases would be quite simple and consume much less time (probably within a day) than nucleotide sequence which takes a week. However, major parts of this procedure, such as PCR amplification, endonuclease digestion and electrophoresis each of which takes around 1 to 3 hrs, can not be shortened. Then, this technique will not be a practical way especially when rapid species identification at dock-side and/or subsequent analysis of large numbers of sample are needed. For rapid species identification, utilizing species-specific monoclonal antibody would have numerous advantages (Rossi *et al.*, 1991). Alternatively, construction of species-specific oligonucleotide probes may be the prime consideration with this issue as expected by Beckenbach (1991). However, the PCR-RFLP analysis may secure the results obtained by these methods or could be a temporal way for species identification until these species specific probes fully approve themselves.

The data obtained by RFLP analysis may provide information on genetic relationships between species and on genetic stock structure within species. Present study proved that RFLP analysis on PCR products is much simpler than conventional mtDNA or nucleotide sequence analyses and suited for large-scale surveys requiring analysis of large numbers of samples. Conventional mtDNA analysis has required mitochondrial-rich tissues such as ovary and liver which are usually gutted out on offshore. Even when such tissues could be obtained, the conventional methods might run out of the valuable DNA sample before completing sufficient analysis. PCR amplification enabled us to proceed with routine collection of tiny muscle tissue at landing site. Further, successful gene amplification using ethanol preserved fish embryo or larvae (Chow *et al.*, 1993) may allow us to carry out unambiguous species identification of very small ichthyo-plankton sample preserved in ethanol. It would be also possible to compare genetic population structure of embryo or larvae collected in spawning ground and that of adult or sub-adult captured at high sea. It might be questioned that the PCR products may be too short for RFLP analysis to detect enough genetic information. Utilizing 4-base endonuclease as in the present study and amplification and analysis of other domeins of mtDNA molecule may overcome this problem. In fact, using

longer DNA fragment of mitochondrial ATPase gene amplified by PCR, high polymorphisms on the restriction fragment length within and between tuna species have been observed (Chow and Inoue, 1993).

5. ACKNOWLEDGEMENTS

I greatly indepted to Drs. J. X. Hartman, FAU, M. I. Farber and E. D. Prince, NMFS Miami Laboratory, and M. Yesaki, IPTP, for sharing their invaluable billfish specimen. I also thank Dr. P. J. Walsh, University of Miami, who kindly allowed me to use his laboratory for DNA extraction. Thanks are also due to Mr. I. Warashina and Dr. Y. Nishikawa, NRIFSF, for their kind help on sample collection and to Dr. Z. Suzuki for reading this manuscript. This research was supported by the Japanese Science and Technology Agency (JSTA).

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Table 1. Billfish species used and the collection sources.

Species	FAO name (abbreviation)	Sources and number of specimens	
Istiophorus albicans	Atlantic sailfish (ASA)	Western Atlantic	NMFS (12), FAU (3)
I. platypterus	Indo-Pacific sailfish (PSA)	Indian Ocean	NRIFSF (1)
Makaira indica	Black marlin (BLM)	Western Pacific	NRIFSF (12)
M. mazara	Indo-Pacific blue marlin (PBM)	Western Pacific	NRIFSF (19)
M. nigricans	Atlantic blue marlin (ABM)	Western Atlantic	FAU (14)
Tetrapturus albidus	Atlantic white marlin (AWM)	Western Atlantic	NMFS (1), FAU (13)
T. angustirostr	is Shortbill spearfish (SBS)	Central Pacific	NRIFSF (5)
T. audax	Striped marlin (PSM)	Western Pacific	NRIFSF (21)
T. pfluegeri	Longbill spearfish (LBS)	Western Atlantic	NMFS (1)
Xiphias gladius	Swordfish (SWF)	Western Pacific	NRIFSF (12)
		Indian Ocean	IPTP (12)
		Western Atlantic	NMFS (1)

FAU: Florida Atlantic University
IPTP: Indo Pacific Tuna Development and Management Programme
NMFS: National Marine Fisheries Service
NRIFSF: National Research Institute of Far Seas Fisheries

Table 2. Thirteen restriction endonucleases used in this study.

Enzyme	Sequence	Manufacturers
Alu I	AG^CT	GIBCO BRL
Bsa JI	^CCNNGG	New England Biolab
Bst UI	CG^CG	New England Biolab
Dde I	C^TNAG	GIBCO BRL
Hae III	GG^CC	GIBCO BRL
Hha I	GCG^C	GIBCO BRL
Hinf I	G^ANTC	GIBCO BRL
Mbo I	^GATC	GIBCO BRL
Msp I	C^CGG	GIBCO BRL
Rsa I	GT^AC	GIBCO BRL
Sau 96I	G^GNCC	New England Biolab
Scr FI	CC^NGG	New England Biolab
Taq I	T^CGA	GIBCO BRL

Table 3. Fragment sizes produced by restriction endonuclease digestion of mitochondrial cytochrome b gene amplified by PCR.

Restriction endonuclease	Type	Fragment sizes
Alu I	A	350
	B	315
	C	310
Bsa JI	A	350
	B	265, 85
	C	230, 120
	D	195, 120
Bst UI	A	325
	B	250, 100
Dde I	A	325
	B	295
	C	230
HaeIII	A	155, 120
	B	140, 130
	C	130, 100
Hha I	A	190, 160
Hinf I	A	200, 150
Mbo I	A	350
Msp I	A	350
	B	210, 140
Rsa I	A	350
Sau 96I	A	350
	B	240, 110
Scr FI	A	350
	B	220, 120
Taq I	A	350
	B	185, 165
	C	165, 130

Table4. Composite genotypes of ten billfish species. Genotypic descriptions present 13 columns representing 13 endonucleases: 1: AluI; 2: BsaJI; 3: BstUI; 4: DdeI; 5: HaeIII; 6: HhaI; 7: HinfI; 8: MboI; 9: MspI; 10: RsaI; 11: Sau96I; 12: ScrFI; 13: TaqI.

Species	No. Individual	Composite genotype												
		1	2	3	4	5	6	7	8	9	10	11	12	13
I. albicans (ASA)	14	C	C	A	C	A	A	A	A	B	A	A	B	B
	1	C	C	A	C	A	A	A	A	B	A	A	A	B
I. platypterus (PSA)	1	C	C	A	C	A	A	A	A	A	A	A	B	B
M. indica (BLM)	12	C	A	A	C	A	A	A	A	B	A	A	A	C
M. mazara (PBM)	19	A	A	A	C	A	A	A	A	A	A	A	A	C
M. nigricans (ABM)	14	A	A	A	C	A	A	A	A	A	A	A	A	C
T. albidus (AWM)	14	C	B	A	B	A	A	A	A	B	A	A	B	C
T. angustirostris (SBS)	5	A	A	A	C	A	A	A	A	B	A	B	B	B
T. audax (PSM)	21	C	B	A	B	A	A	A	A	B	A	A	B	C
T. pfluegeri (LBS)	1	A	A	A	C	A	A	A	A	B	A	A	B	B
X. gladius (SW)	23	B	D	B	A	C	A	A	A	A	A	A	A	A
	2	B	D	B	A	B	A	A	A	A	A	A	A	A

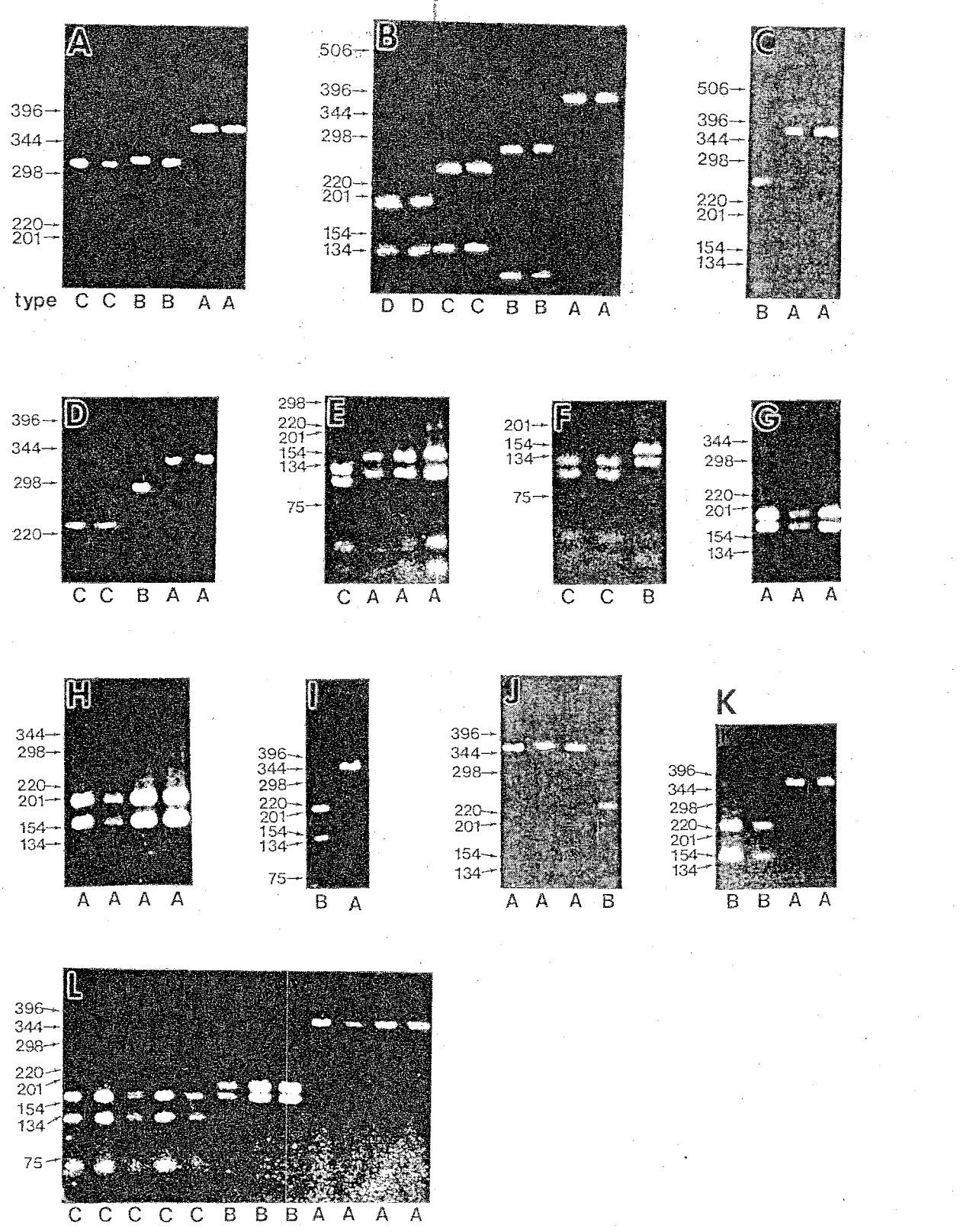

Figure 1. Representative restriction patterns of 350bp mitochondrial cytochrome *b* gene fragment of billfish species. Figures on the left side of each photograph represent positions of size standard (1kb ladder, BRL). A: *Alu* I); B: *Bsa* JI; C: *Bst* UI; D: *Dde* I; E and F: *Hae* III; G: *Hha* I; H: *Hinf* I; *Msp* I; J: *Sau* 96I; K: *Scr* FI; L: *Taq* I.