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EPIC-PCR for ribosomal protein gene introns: a case study in *Thunnus* tuna species

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Abstract

Thirty-four exon-primed intron-crossing PCR (EPIC-PCR) primer pairs designed to amplify ribosomal protein gene introns were tested in eight individuals from four *Thunnus* tuna species. Only two pairs failed to amplify fragments for all individuals. Whenever amplicons were observed, no apparent fragment length difference between species was observed. Possible allelic variation due to fragment length polymorphism was observed at 13 loci.

Key words: amplicon size; ribosomal protein gene; *Thunnus*; intron

Introduction

PCR amplification of introns using conserved primers designed in exon sequences was pioneered by Lessa (1992) and subsequently named exon-primed intron-crossing PCR (EPIC-PCR) (Palumbi and Baker 1994). PCR primers designed in highly conserved exon regions would be of universal utility. Sequence variation in introns within species may be a simple genetic marker for investigating population structure (Nakadate et al. 2005; Chow et al. 2007), and that between species may be used for species identification and phylogenetic analysis (López-Fernández et al. 2010; Boscari et al. 2024). Genes encoding ribosomal proteins (RPs) are ideal for designing universal primers because RPs are among the most highly conserved proteins in eukaryotes (Ban et al. 2014). In addition, each protein consisting ribosomal subunits is typically encoded by a single gene (Kenmochi et al. 1998).

Dozens of universal primer candidates have been proposed to amplify ribosomal protein gene introns (Chow and Yanagimoto 2015; Chow et al. 2016). Some primer pairs have been confirmed to successfully amplify a single fragment even in distantly related fish species (Chow et al. 2016), and we have attempted PCR using the primer pairs to investigate their general utility for closely related species and to find amplicon length polymorphism within and between species. Here, we report the results of testing some of these primers using four tuna species of the genus *Thunnus*.

Materials and Methods

Crude DNA samples from eight individuals of four *Thunnus* tuna species were obtained from our laboratory collection. The samples consisted of four individuals of Pacific bluefin tuna (*T. orientalis*) (PBF), one Atlantic bluefin tuna (*T. thynnus*) (ABF), one southern bluefin tuna (*T. maccoyii*) (SBF), and two bigeye tuna (*T. obesus*) (BET). One of the two bigeye tunas was α type from Atlantic and the other was β type from Pacific (see Chow et al. 2000). The same set of samples was used for all PCR primer pairs. Thirty-four primer pairs used for PCR amplification are presented in Table 1, of which 30 primer pairs were confirmed to amplify fragments in Pacific bluefin tuna or skipjack tuna (*Katsuwonus pelamis*) in previous studies (Chow and Yanagimoto 2015; Chow et al. 2016). The composition of the PCR mixture follows Chow and Yanagimoto (2015). The same reaction con-

Table 1. Primer pairs used in this study and the results of PCR amplification.

RP	primer			Number of amplified fragments			Fig. 1
	ID	forward	reverse	bluefin tuna**	skipjack tuna*	this study	
L3	L3exA-B*	AF	BR		2	1	A
	L3exB-C*	BF	CR		1	1	B
	L3exD-E*	DF	ER		1	1–2	C
L8	L8ex2-3**	2F	3R	1		1–2	J
	L8exB-C*	BF	CR		1	1	G
L9	L9exC-D*	CF	DR		3	0–3	H
L11	L11exA-B*	AF	BR		3	1	I
	L11exB-C*	BF	CR		1	1	P
L12	L12exD-E**	DF	ER	1		1	K
L17	L17exA-B*	AF	BR		1	1–2	Q
L21	L21ex2-3**	2F	3R	not examined		0	O
	L21ex4-5**	4F	5R	2?		1–2	E
L24	L24exB-C*	BF	CR		3+	0-1	M
L30	L30ex3-4**	3F	4R	3		1	L
L37	L37ex2-3**	2F	3R	2		1–3	D
P0	P0exA-B**	AF	BR	1		1	AA
P1	P1ex2-3**	2F	3R	3		0–2	AB
P2	P2ex2-3**	2F	3R	not examined		0-1	AD
S2	S2exB-C**	BF	CR	not examined		1	AE
	S2exC-D*	CF	DR		1	1	AG
S3	S3ex1-2**	1F	2R		1	0-1	AH
	S3ex2-3**	2F	3R	2		1–2	AC
	S3ex3-4*	3F	4R		1	1	R
	S3ex4-5*	4F	5R		1	1–2	S
S6	S6ex2-3**	2F	3R	1		1	F
S7	S7ex6-7**	6F	7R	1		1	U
S8	S8ex3-4**	3F	4R	not examined		1	AF
	S8ex4-5**	4F	5R	1		1–2	V
S9	S9ex4-5**	4F	5R	1		0-1	W
S14	S14exA-B*	AF	BR		1	1–2	T
S17	S17exB-C*	BF	CR		2	1	N
S23	S23exA-B*	AF	BR		3	1	X
	S23exB-C*	BF	CR		3	1–2	Y
S27	S27exA-B*	AF	BR		1	0	Z

See Chow and Yanagimoto (2015)* and Chow et al. (2016)** for primer information.

dition was used for all primer pairs, in which the reaction mixtures were preheated to 94 °C for 4 min, followed by 35 amplification cycles (denaturing at 94 °C for 30 s, annealing at 56 or 57 °C for 30 s, and extension at 72 °C for 50 s), with a final extension at 72 °C for 7 min. PCR products were electrophoresed on 1.5 % agarose gels (UltraPure Agarose; Invitrogen Corporation, Carlsbad, CA, USA), stained with ethidium bromide, and photographed.

Results

Size and number of amplicons, variation within and between species are summarized below.

L3exA-B (Fig. 1A): very strong amplification of a single fragment of approximately 470 bp fragment in all individuals, no length variation within and between species.

L3exB-C (Fig. 1B): very strong amplification of a single fragment of approximately 420 bp fragment in all individuals, no length variation within and between

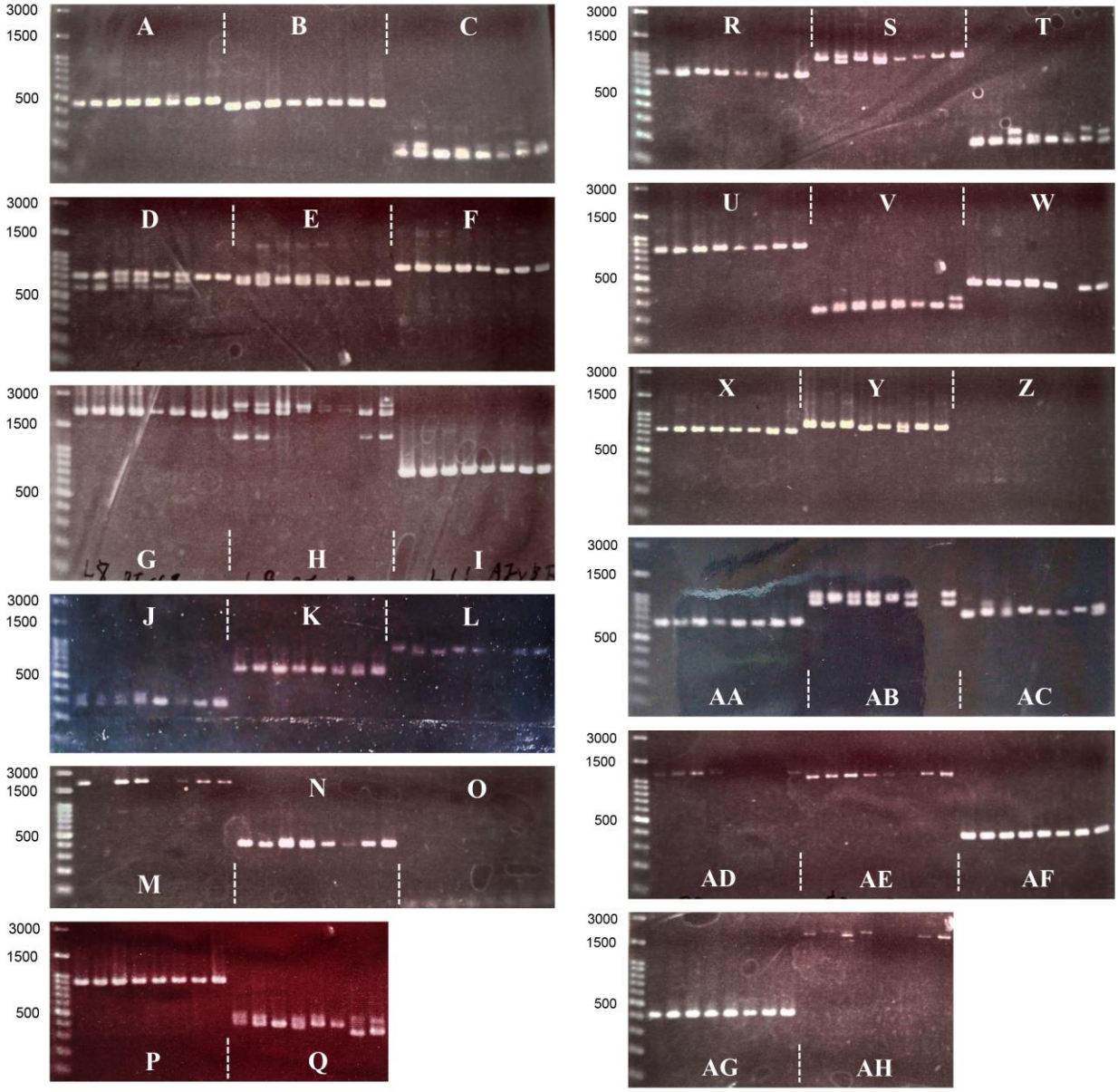

Fig. 1. Agarose gel electrophoresis images of the PCR amplicons using 34 primer pairs for ribosomal protein (RP) gene introns. Left most ends in each gel are the size marker with size in bp. Samples from left to right are four Pacific bluefin tuna (*Thunnus orientalis*) (PBF), one Atlantic bluefin tuna (*T. thynnus*) (ABF), one southern bluefin tuna (*T. maccoyii*) (SBF), one α type bigeye tuna (*T. obesus*) (BETα) from Atlantic, and one β type bigeye tuna from Pacific (BETβ). See Table 1 for primer information.

species.

L3exD-E (Fig. 1C): weak to very strong amplification of approximately 170−200 bp fragments, one to two fragments, allelic variation?

L37ex2-3 (Fig. 1D): strong amplification of approximately 590−720 bp fragments in all individuals, two to three fragments in PBF, two fragments in ABF, three fragments in SBF, one fragment in BET, allelic variation?

L21ex4-5 (Fig. 1E): strong amplification of approximately 600−700 bp fragments, one to two fragments, allelic variation?

S6ex2-3 (Fig. 1F): strong to very strong amplification of approximately 800 bp fragment in all individuals, one fragment, no length variation within and between species.

L8exB-C (Fig. 1G): strong to very strong amplification of approximately 1,900 bp fragment in

all individuals, no length variation within and between species.

L9exC-D (Fig. 1H): weak to strong amplification of approximately 1,200−2,000 bp fragments, one to three fragments in PBF, faint amplicon in ABF and SBF, two to three fragments in BET, allelic variation?

L11exA-B (Fig. 1I): very strong amplification of approximately 720 bp fragment in all individuals, no length variation within and between species.

L8ex2-3 (Fig. 1J): fait to weak amplification of approximately 290−310 bp fragment, two fragments in PBF, one fragment in the others, allelic variation?

L12exD-E (Fig. 1K): weak amplification of approximately 600 bp fragment in all individuals, no length variation within and between species.

L30ex3-4 (Fig. 1L): fait to weak amplification of approximately 800 bp fragment in all species, no length variation within and between species.

L24exB-C (Fig. 1M): No amplicon was observed in one PBF and ABF, weak to strong amplification of approximately 1,800 bp fragment, no length variation within and between species.

S17exB-C (Fig. 1N): weak to very strong amplification of approximately 410 bp fragment, no length variation within and between species.

S21ex2-3 (Fig. 1O): no amplicon in all species.

S11exB-C (Fig. 1P): weak amplification of approximately 900 bp fragment, no length variation within and between species.

S17exA-B (Fig. 1Q): weak amplification of approximately 300−450 bp fragments, one to two fragments, allelic variation?

S3ex3-4 (Fig. 1R): weak to very strong amplification of approximately 720 bp fragment, no length variation within and between species.

S3ex4-5 (Fig. 1S): weak to very strong amplification of approximately 900−1,000 bp fragments, one to two fragments in PBF, one fragment in the others, allelic variation?

S14exA-B (Fig. 1T): strong to very strong amplification of approximately 170−210 bp fragments, one to two fragments in PBF, one fragment in ABF and SBF, two fragments in BET, allelic variation?

S7ex6-7 (Fig. 1U): strong to very strong amplification of approximately 800 bp fragment, no length variation within and between species.

S8ex4-5 (Fig. 1V): strong to very strong amplification of approximately 250−300 bp fragments, one fragment in BETα and two fragments in BETβ, allelic variation?

S9ex4-5 (Fig. 1W): no amplicon in SBF, strong to very strong amplification of approximately 450 bp fragment in the other, no length variation within and between species.

S23exA-B (Fig. 1X): strong to very strong amplification of approximately 750 bp fragment, no length variation within and between species.

S23exB-C (Fig. 1Y): very strong amplification of approximately 790−810 bp fragments, allelic variation?

S27exA-B (Fig. 1Z): no amplicon in all individuals.

P0exA-B (Fig. 1AA): strong amplification of approximately 650 bp fragment in all individuals, no length variation within and between species.

P1ex2-3 (Fig. 1AB): no amplicon in BETα, strong amplification of approximately 900−1,000 bp fragments in the others, allelic variation?

S3ex2-3 (Fig. 1AC): weak to strong amplification of approximately 720−760 bp fragments, allelic variation?

P2ex2-3 (Fig. 1AD): faint to weak amplification of approximately 1,200 bp fragment in PBF and BETβ, no amplicon in the others.

S2exB-C (Fig. 1AE): faint to strong amplification of approximately 1,000 bp fragment, no length variation within and between species.

S8ex3-4 (Fig. 1AF): very strong amplification of approximately 380 bp fragment, no length variation within and between species.

S2exC-D (Fig. 1AG): very strong amplification of approximately 410 bp fragment, no length variation within and between species.

S3ex1-2 (Fig. 1AH): no amplicon in ABF and SBF, faint to weak amplification of approximately 1,700−1,800 bp fragments in the others, allelic variation?

Discussion

Only two out of 34 primer pairs tested in this report failed to obtain amplicon in all individuals, supporting cross-species efficacy and transferability of the EPIC-PCR approach. No apparent amplicon length variation was observed between tuna species, indicating little utility of these loci for species discrimination in terms of amplicon length polymorphism. On the other hand, possible allelic variation observed at 13 loci may be useful for population genetic analysis. Amplification efficiency by some primer pairs was observed to vary within and between species and among fragments, to which differences in primer annealing efficiency, allele length, and template DNA secondary structures may be responsible (Buchan et al. 2005; Martins et al. 2011). These factors leading to false genotyping have to be eliminated as possible prior to actual assay. Furthermore, possible allelic variation observed at several loci must be substantiated using a large number of individuals.

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