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ORIGINAL RESEARCH

Universal PCR primers for ribosomal protein gene introns of fish

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Abstract Human ribosomal protein (RP) gene sequences with respect to intron/exon structures and corresponding cDNA or genomic data of fish species were obtained from the GenBank database. Based on conserved exon sequences, 128 primer pairs for 41 genes were designed for exon-primed intron-crossing (EPIC) polymerase chain reaction (PCR). In reference to the draft genome sequences of the Pacific bluefin tuna (*Thunnus orientalis*), 12 primer pairs expected to amplify introns of the bluefin tuna with lengths of 500–1000 bp were selected and applied to six distantly related fish species belonging to the Orders Clupeiformes, Tetraodontiformes, Pleuronectiformes, Perciformes, Scorpaeniformes, and Anguilliformes. PCR amplification was observed for at least four species in each primer pair, and all fragments were larger than those expected for intronless amplification. Single fragment amplification was observed for at least seven primer pairs per species. Fragment sizes of the bluefin tuna for nine primer pairs corresponded to those expected from the genomic data. Thus, our primer pairs are potentially applicable to a wide variety of fish species and serve as an initial step for isolating single-copy nuclear DNA sequences.

Keywords Universal primers · Ribosomal protein genes · Intron · Single copy nuclear loci · Teleostei

Introduction

Because introns are a good source to find DNA polymorphisms in the eukaryotic genome, exon-primed intron-crossing (EPIC) polymerase chain reaction (PCR) assays have been developed for genetic analyses (Lessa 1992; Palumbi and Baker 1994; Corte-Real et al. 1994; Chow and Hazama 1998; Chow 1998; Quattro and Jones 1999; Hassan et al. 2002; Jarman et al. 2002; Chow and Nakadate 2004; Nakadate and Chow 2008; Pinho et al. 2010; Jennings and Etter 2011; Chow et al. 2015). Polymorphisms detected in introns have been used for genetic population analysis (Palumbi and Baker 1994; Chow and Takeyama 2000; Williams et al. 2002; Nohara et al. 2003; Touriya et al. 2003; Nakadate et al. 2005; Berrebi et al. 2005; Atarhouch et al. 2007; Chow et al. 2007; Yanagimoto et al. 2012), investigations of interspecific hybridization (Daguin et al. 2001; Pacheco et al. 2002; Yao et al. 2015), and phylogenetic analysis (DeBry and Seshadri 2001; Berrebi et al. 2005; Creer 2007; Near and Cheng 2008; He et al. 2008; Pinho et al. 2010).

The universal specificity of primers may depend on the conservation of exon sequences whereas the efficiency of isolating a single copy of the target nuclear genome may be a function of the number of duplicate

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gene copies, paralogs, and/or pseudogenes. Ribosomal proteins (RPs), which play fundamental roles in protein synthesis, are the most highly conserved proteins among eukaryotes, and each protein is typically encoded by a single gene (Wool 1979; Wool et al. 1995; Kenmochi et al. 1998; Yoshihama et al. 2002). Therefore, RP genes may be particularly suitable for designing universal primers. In spite of this potential, the number of studies reporting universal primers for the amplification of RP gene introns of aquatic animals is small (Chow and Hazama 1998; Pinho et al. 2010; Chow et al. 2015). Here we report the development of universal primer pairs to amplify 128 intron regions of 41 RP genes in fishes, which may be potentially applicable to distantly related fish species.

Materials and methods

The intron/exon structures of human RP genes have been reported by Kenmochi et al. (1998) and Yoshihama et al. (2002), and the nucleotide sequences are available in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). Reference cDNA data of *Danio rerio*, *Ictalurus punctatus*, *Salmo salar*, *Takifugu rubripes*, and *Paralichthys olivaceus*, corresponding to orthologs of the human RP genes were obtained from the GenBank database. These fish species are taxonomically distant one another and offer abundant data set of ribosomal protein gene. The Orders to which these fish species belong were Cypriniformes, Siluriformes, Salmoniformes, Tetraodontiformes, and Pleuronectiformes, respectively, and we anticipated that primers designed using conserved sequences among these distant fish species may have a universal specificity. The draft genome of the Pacific bluefin tuna, *Thunnus orientalis* (accession nos. BADN01000001–BADN01133062 in the DNA Data Bank of Japan) (Nakamura et al. 2013) was also utilized. The Pacific bluefin tuna belongs to the Order Perciformes. Multiple sequence alignments were performed using ClustalX version 1.83 (Thompson et al. 1997), and conserved exon regions were visually inspected. Rules for primer design included; primer length of 20 nucleotides, less than two degenerate bases, a moderate GC content (approximately 50 %), no degenerated bases within last two positions, and avoiding presence of the primer secondary structure produced by inter- and intra-molecular interactions. Furthermore, more than 20 bp distances was placed between the splicing site and 3′-end of at least one of a primer in each pair, which may be used to authenticate amplicons by nucleotide sequence analysis. All these procedures were performed by eyes.

Because intron size cannot be predicted and there may be considerable variations even between closely related taxa, draft genome sequences of the Pacific bluefin tuna were used to select primer pairs for PCR testing. A total of 12 primer pairs expected to amplify RP gene introns of the Pacific bluefin tuna with lengths of 500–1000 bp were selected for ease of sizing, and PCR amplification was attempted using template DNA of the Japanese pilchard (*Sardinops melanostictus*), the Japanese puffer (*Takifugu rubripes*), starry flounder (*Platichthys stellatus*), the Pacific bluefin tuna (*T. orientalis*), broadbanded thornyhead (*Sebastolobus macrochir*), and the Japanese eel (*Anguilla japonica*), all derived from the tissue collection in our laboratory. These fishes belong to the Orders Clupeiformes, Tetraodontiformes, Pleuronectiformes, Perciformes, Scorpaeniformes, and Anguilliformes, respectively, and used to evaluate universal specificity of the primer pairs selected. PCR amplification was carried out in a 12 μL final volume containing 1 μL of template DNA (10–50 ng/μL), 1.2 μL of 10 × reaction buffer (containing 20 mM $MgCl_2$), 1 mM of each deoxynucleotide triphosphate, 0.4 μM of each primer, and 0.5 U of EX *Taq* polymerase (Takara Bio, Inc., Shiga, Japan). The same reaction conditions were 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 °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) and stained with ethidium bromide. The gel images visualized on transilluminator were captured by CCD camera.

Results and discussion

Based on the relatively high degree of conservation of the exon sequences among distant reference fish species and human, a total of 128 primer pairs for the amplification of 41 RP genes were designed for EPIC PCR. Among the 41 RP genes listed in Table 1, whole or partial sequences of 29 genes (Table 1, dagger) were

Table 1 Sequences of 128 primer pairs for 41 ribosomal protein (RP) genes

RP	Pair	Forward	5′-3′	*Tm*	Reverse	5′-3′	*Tm*
L3†	1	L3ExAF	GGATACAARGCYGGCATGAC	62.0	L3ExBR	TCCACCACYTCCTTYTTGTT	60.9
L3†	2	L3ExBF	GCACRTCAGTGATGARTGCA	63.5	L3ExCR	TTGGTRAARGCCTTCTTCTT	57.1
L3†	3	L3ExCF	GGCAAGAARCAGCTGGAGAA	64.3	L3ExDR	CTCAGRGGCAGCAGRCGCAT	68.1
L3†	4	L3ExDF	CAGGAYGAGATGATYGACGT	60.1	L3ExER	AGCTTCTTBGTGTGCCAACG	65.2
L8†	5*	L8Ex2F	CAYATTGACTTCGCTGARCG	60.8	L8Ex3R	TTGCCGCAGTAGATRAACTG	60.4
L8†	6	L8Ex3F	CAGTTYATCTACTGCGGCAA	60.4	L8Ex4R	GGCTTCTCCTCCAGRCAGCA	66.9
L8†	7	L8Ex4F	ATCTCYTCTGCMAACAGAGC	55.3	L8Ex5R	TTCARGATGGGTTTGTCAAT	60.2
L8†	8	L8Ex5F	GACAAACCCATCYTGAAGGC	63.4	L8Ex6R	ATGTGCTGRTGGTTRCCACC	62.3
L9†	9	L9Ex2F	ATGAAGACYATTCTCAGYAA	47.5	L9Ex3R	GGTTRATGTGGTTGAACTCC	58.3
L9†	10	L9Ex3F	TTCAACCACATYAACCTGGA	60.4	L9Ex4R	CACCATTTWTCCACACGCAG	63.9
L9†	11	L9Ex4F	GTCCAGAACATGATYAAGGG	58.4	L9Ex5R	RAAGTTYCTGATCTCCACCA	57.2
L9†	12	L9Ex5F	CTGGTGGAGATCAGRAACTT	58.3	L9Ex6R	TTWGACACCWGCTCAATATC	54.2
L9†	13	L9Ex6F	GACGAGTTGRTTCTGGARGG	61.2	L9Ex7R	ACWGTGGTGGCYTGCTGGAT	67.0
L12†	14	L12ExAF	TTCATCCGACACCCTCACCA	68.9	L12ExBR	AGGTCCRATTTTGGGGGCCA	71.3
L12†	15	L12ExBF	TGGCCCCCAAAATYGGACCT	71.3	L12ExCR	AGGCCYTTCCAGTCACCGGT	69.1
L12†	16	L12ExCF	AAGCTGACCATCCAGAACAG	61.9	L12ExDR	TTCTTCCTGTCACGRGGAGG	64.3
L12†	17*	L12ExDF	CCTCCYCGTGACAGGAAGAA	64.3	L12ExER	CCCAGAATCTCCTTRATGGT	59.4
L17†	18	L17Ex2F	TCGCTCGACCCVGAGAACCC	71.0	L17Ex3R	TTAAAGTGRACACGAAGRTT	53.9
L17†	19	L17Ex3F	TCCAACCTTCGTGTYCACTT	62.1	L17Ex4R	TATGCATRCCYTTGATGGCCT	63.0
L17†	20	L17Ex4F	AAGCACCAGTGTGTYCCCTT	63.5	L17Ex5R	AGCATGTGVAGGAGGAACTC	60.8
L17†	21	L17Ex5F	CTBCACATGCTGAAGAACGC	63.6	L17Ex6R	AGAGAGTCCACATCYAAACC	54.6
L17†	22	L17Ex6F	AAGTYTCTCAGAAGAAAAAG	51.9	L17Ex7R	ATTTACTCCCGWGCCATAAG	59.6
L18	23	L18Ex3F	AATGCTCCCTTCAACARGGT	62.4	L18Ex4R	TGAATYCTGACATCATCAGT	55.3
L21†	24	L21Ex2F	CAGGCCCTTCCGCAAGCATG	74.0	L21Ex3R	CCYTTCTTRTAGATGCGCAT	58.4
L21†	25	L21Ex3F	ATGCGCATCTAYAAGAARGG	58.4	L21Ex4R	TAGCAYTTATGAGGCATRCC	56.7
L21†	26*	L21Ex4F	GTMGGCATCATTGTCAACAA	62.0	L21Ex5R	TCYCTGCTCTTTGAGTGCTT	59.5
L21†	27	L21Ex5F	AGCGTRTCAARGAGAACGAG	58.7	L21Ex6R	TTCTTRGTGCTGACGAAGTG	58.2
L24†	28	L24Ex2F	CTGTGCAGTTTYAGYGGGTA	57.8	L24Ex3R	AAGGCAGACTCRCAYTTGGC	64.1
L24†	29	L24Ex3F	ARCACAAGAAGGGCCAGTCT	62.6	L24Ex4R	CTTCTGGTTCCTCTTGGCCA	66.5
L24†	30	L24Ex4F	GCCAAGAGGAACCAGAAGCC	67.2	L24Ex5R	TCTTSGCCTCCTTKGCAGCC	70.9
L24†	31	L24Ex5F	GGCTGCMAAGGAGGCSAAGA	70.9	L24Ex6R	TTCATGGGCTTRGCRATCTT	61.1
L26†	32	L26Ex2F	TACAACGTGMGSTCCATGCC	63.5	L26Ex3R	TGCTGGCCTTTRTAGTGTCC	61.6
L26†	33	L26Ex3F	AACGGAACCACWGTCCAYG	63.5	L26Ex4R	TCYTCCTTGTATTTGCCCTT	60.1
L27†	34	L27Ex2F	CTGGCTGGACGYTACKCCGG	68.9	L27Ex3R	TAAGGRCGGTCWGCGGTGCC	71.6
L27†	35	L27Ex3F	CHRCCATGGGCAAGAAGAAG	64.4	L27Ex4R	TTGTCCAGAGGAATRTCAAC	56.5
L27†	36	L27Ex4F	CCAAGGTCAAGTTTGAGGAG	61.2	L27Ex5R	CKGAGYTTCTGGAAGAACCA	59.4
L30†	37	L30Ex2F	AAAATGGTGGCCGCAAAGAA	68.4	L30Ex3R	TGGGACTGYTTGTAKCCCAG	62.4
L30†	38*	L30Ex3F	TCATCCTGGCCAACAACTGC	68.5	L30Ex4R	TTCCACTGTARTGRTGGACA	58.9
L30†	39	L30Ex4F	AAATACTWCAGGGTSTGCAC	56.5	L30Ex5R	GGCATRCTYCTGATGATGTC	57.0
L31†	40	L31Ex2F	AACRTSCACAAGCGCATCCA	69.9	L31Ex3R	RGGAGTKCCCATCTCCTTCA	62.6
L31†	41	L31Ex3F	TTCGCMRTGAAGGAGATGGG	67.2	L31Ex4R	CTCRTTRCGCTTCCTGGACA	62.8
L31†	42	L31Ex4F	CTGTCCAGGAAGCGYAAYGA	62.8	L31Ex5R	TCATCAACATTGACWGTCTG	57.0
L32†	43	L32Ex2F	AAGAGRACYAAGAAGTTCAT	48.6	L32Ex3R	GACYCTGTTRTCAATACCTC	49.8
L32†	44	L32Ex3F	AAGACCAARYACATGCTGCC	61.5	L32Ex4R	TTGTGRGCRATCTCTGCACA	63.3
L34	45	L34Ex2F	GCCTCCAACAARACYAGGCT	62.1	L34Ex3R	AGGTAYACRATGCGGTTACC	57.9
L34	46	L34Ex3F	TGCCCYGGYAGACTGCGTGG	70.7	L34Ex4R	TTKGTCTTTGARAGCCTCAT	58.9
L34	47	L34Ex4F	CCTATGGYGGTKCCATGTGT	62.1	L34Ex5R	TRAGGAAAGCACGCTTGATC	61.8
L36†	48	L36Ex3F	GCCATGGAGYTGCTGAARGT	64.1	L36Ex4R	AGCYTTCCTCATGGCRGCCA	69.3

Table 1 continued

RP	Pair	Forward	5′-3′	*Tm*	Reverse	5′-3′	*Tm*
L37†	49*	L37Ex2F	TCCWCCTGCGGCAAGTGTGG	73.1	L37Ex3R	TTGGCCTTRGCRCTCCAGTT	63.6
L37†	50	L37Ex3F	AACTGGAGTGCYAAGGCYAA	58.2	L37Ex4R	TGTTCCYTCACGGAAWCCAT	64.2
L39†	51	L39Ex1F	CWACYACCGCCATAGTGGTG	62.9	L39Ex2R	TTRATCCTGAARGTCTTGTG	54.3
L39†	52	L39Ex2F	GAARACTGGCAACAARATCA	60.1	L39Ex3R	CCCAGCTTGGTYCTTCTCCA	66.5
P0†	53*	P0ExAF	ATGATGCGYAARGCCATCCG	66.8	P0ExBR	GYAAGRTCCTCCTTGGTGAA	58.1
P0†	54	P0ExBF	TTYGTSTTCACCAAGGAGGA	62.2	P0ExCR	ATKGCWCCAGCACGGGCAGC	74.8
P0†	55	P0ExCF	GGGGMACCATTGAAATCYTG	63.0	P0ExDR	AGSAGCGTGGCYTCGCTGGC	76.1
P0†	56	P0ExDF	GGTGCTTGACATCACWGAGG	63.4	P0ExER	GGGTAGCCRATCTSCAGACA	64.0
P1†	57	P1Ex1F	TCTGTSTCCGARCTCGCCTG	68.0	P1Ex2R	AGAGCRTTCAGYTTGTCCTC	58.4
P1†	58*	P1Ex2F	AAYGCYCTGATCAAGGCTGC	64.4	P1Ex3R	CAGATCAGRCTRCCGATGTC	57.1
P2†	60	P2Ex2F	CGTTACGTKGCYGCTTACCT	61.8	P2Ex3R	TCTTTTCCATTTARYTCACT	52.9
P2†	61	P2Ex3F	AAAGACATCRATGARGTCAT	53.3	P2Ex4R	ACAGCACCACCKGCYGGCAC	71.2
P2†	62	P2Ex4F	GTGCCRGCMGGTGGTGCTGT	71.2	P2Ex5R	AATCCCATGTCRTCATCRGA	60.3
S2†	63	S2ExAF	GACGCCGGTGGTAGAGGAGG	70.2	S2ExBR	GACTTGATCTTCATGTCCTT	55.5
S2†	64	S2ExBF	AAGGACATGAAGATCAAGTC	55.5	S2ExCR	TGGACAGGCATGATCTTCAG	63.9
S2†	65	S2ExCF	CTGAAGATCATGCCTGTCCA	63.9	S2ExDR	ACYTCTTTRGAGCACTTCAC	52.6
S2†	66	S2ExDF	CTGCTSATGATGGCTGGTAT	62.7	S2ExER	TTCCAGAGATCAGGGGTCAG	64.2
S3†	67	S3Ex1F	AAGATGGCGGTGCAAATCTC	66.2	S3Ex2R	AACTCRTTCAGCTCGGCYTTG	65.4
S3†	68*	S3Ex2F	GAGGTRCGTGTGACWCCAAC	60.5	S3Ex3R	TTCTCTCCCAGMACATTCTG	59.4
S3†	69	S3Ex3F	TGACCGCTGTGGTYCAGAAG	66.5	S3Ex4R	AGACCACGAGTSGCWACCTT	66.1
S3†	70	S3Ex4F	GCAGAGTCTYTGCGYTACAA	60.6	S3Ex5R	AACCKCAGMACACCATAGCA	59.7
S3†	71	S3Ex5F	GATGATCCACAGYGGAGACC	62.0	S3Ex6R	AGCATGATCTTMACCTTGAT	55.2
S4†	72	S4Ex2F	CATTGGATGCTKGACAAACT	60.1	S4Ex3R	AYTCCCTCAGYTTGTGGGGA	65.5
S4†	73	S4Ex3F	ATCAARATYGATGGCAAGGT	60.7	S4Ex4R	ACCWGTYTTCTCGATGCTGA	62.4
S4†	74	S4Ex4F	TTTRCWGTTCACCGCATCAC	61.2	S4Ex5R	TCRGGGTARCGGATGGTGCG	70.2
S4†	75	S4Ex5F	ATYACAGACTTCATCAAGTT	49.9	S4Ex6R	TCYCTGTTGGTGATSACACC	61.2
S4†	76	S4Ex6F	GGTGTSATCACCAACAGRGA	61.2	S4Ex7R	AGGGACACCCAYGGYTTGTT	65.3
S6†	77*	S6Ex2F	GAGAAGCGYATGGCCACAGA	65.2	S6Ex3R	ACACCCTGCTTCATGGGGAA	68.8
S6†	78	S6Ex3F	CGCCAACCTSAGYGTTCTCA	66.5	S6Ex4R	AGCCCRGGAATRTCCTTCTC	61.0
S6†	79	S6Ex4F	ATCCGCAARCTYTTCAACCT	62.3	S6Ex5R	GTAACMAGACGCTGAATCTT	56.0
S6†	80	S6Ex5F	CCMCGTGTSCTGCAGCACAA	71.0	S6Ex6R	TGGCGYTTCTCCTTRGCCTC	65.4
S7†	81	S7Ex1F	TGGCCTCTTCCTTGGCCGTC	72.8	S7Ex2R	AACTCRTCTGGCTTYTCGCC	65.3
S7†	82	S7Ex2F	AGCGCBAAAATAGTGAAGCC	60.0	S7Ex3R	GCCTTCAGGTCAGAGTTCAT	60.8
S7†	83	S7Ex3F	ATGAACTCTGACCTGAAGGC	60.8	S7Ex4R	TGAGGAACRGGMACAAAGAT	58.7
S7†	84	S7Ex4F	GTGCGCGAGYTGGARAAGAA	67.4	S7Ex5R	TTTGTGCGGCTTTTYCTTGT	64.4
S7†	85	S7Ex5F	AACAAGCARAAGCGTCCYAG	61.0	S7Ex6R	CTGGGRAAGACCAGRTCCTC	62.6
S7†	86*	S7Ex6F	CAGAACAAYGTTGAACACAA	57.0	S7Ex7R	TGRAACTCTGGGAATTCAAA	57.1
S8†	87	S8Ex1F	ACTCTTTCYAGCCRGCGCC	63.5	S8Ex2R	CGGTCYTGCGGCGTTTRTGC	72.5
S8†	88	S8Ex2F	ACAAGAARAGRAAGTATGAG	50.0	S8Ex3R	AGRGCACGGTAYTTCTTGTT	57.9
S8†	89	S8Ex3F	AAGAARTACCGTGCTYTGAG	58.4	S8Ex4R	TAGACCACATCRATGATCCT	53.3
S8†	90*	S8Ex4F	GGCMGSAAGAAGGGAGCCAA	70.8	S8Ex5R	TGCWGGAACTGCTCCTCCAG	68.0
S8†	91	S8Ex5F	GAGGAGCAGTTCCWGCAGGG	68.5	S8Ex6R	CCRTCTGCTCKGCCGCACTG	71.7
S9†	92	S9Ex2F	GAGAAGTCYCGTCTYGACCA	59.5	S9Ex3R	AGGGTGAAYTTSACCCTCCA	65.5
S9†	93	S9Ex3F	ACCCYAAGCGTCTSTTTGAA	60.3	S9Ex4R	CCKCACCAGACGCCTGAGCA	71.3
S9†	94*	S9Ex4F	GCCAAGAGYATCCACCAYGC	64.4	S9Ex5R	GGGGATGTTCAMACCTGCTT	65.0
S10†	95	S10ExAF	CCYGAGCTYGCTGACAAGAA	63.3	S10ExBR	CAGGCAAAYTGCTCYTTGAC	62.3
S12	96	S12Ex2F	TCTACAATGGCCGAGGAAGG	66.1	S12Ex3R	TCATGGATGAGKGCRGTCTT	60.9
S12	97	S12Ex3F	ACCGCACTCATCCAYGAYGG	68.3	S12Ex4R	CACARAGATGRGCTTGGCGC	68.1

Table 1 continued

RP	Pair	Forward	5′-3′	*Tm*	Reverse	5′-3′	*Tm*
S12	98	S12Ex4F	TATGTYAAGYTGGTGGAGGC	59.0	S12Ex5R	AGYTTCTTRTTGTCATCAAC	50.4
S12	99	S12Ex5F	CGCAAAGTKGTSGGCTGCAG	71.3	S12Ex6R	CCTYATTTCTTGGATTTGAA	55.8
S13†	100	S13ExAF	AGAAAGGGCTTGTCCCAGTC	64.4	S13ExBR	ATCTGCTCYTTRACATCATC	53.2
S13†	101	S13ExBF	GAGCAGATCTTYAARCTGGC	58.1	S13ExCR	ACCRGTGACGAARCGCACCT	67.0
S13†	102	S13ExCF	TACCACCTMATCAAGAAGGC	58.2	S13ExDR	AGAATCAGGCGGAAYTTGGC	67.2
S14†	103	S14Ex2F	TTCGCMTCCTTCAACGACAC	66.1	S14Ex3R	TCGGCCTTYACCTTCATCCC	67.0
S14†	104	S14Ex3F	ATCACWGCKCTGCACATCAA	62.1	S14Ex4R	CCWGGTCCAGGRGTCTTGGT	65.4
S14†	105	S14Ex4F	GGGGCMCAGTCKGCCCTCAG	71.7	S14Ex5R	GTCACCCCGATCCCGTCAGA	71.9
S15	106	S15Ex2F	GACCAGCTKCTGGACATGTC	62.4	S15Ex3R	GGMGCCTCCTTCTTGGCCTT	67.8
S15	107	S15Ex3F	GGCAAGACYTTCAACCAGGT	63.0	S15Ex4R	GCTTGTARGTGATRGAGAAC	50.3
S16	108	S16Ex1F	CCCCTRCAGTCTGTCCAGGT	63.0	S16Ex2R	GCTACAGCMGTRGCTGTTTT	56.4
S16	109	S16Ex2F	CGGYCACDCTGCAGTACAAG	62.0	S16Ex3R	TGTCCWCCRCCCTTCACACG	69.6
S16	110	S16Ex3F	CGAGTBCGTGTGAAGGGYGG	67.9	S16Ex4R	VACCAGGGCTTTGGAGATGG	67.2
S16	111	S16Ex4F	KCCATCTCCAAAGCCCTGGT	67.9	S16Ex5R	AGCAGRGTYCTGTCGTACTG	57.1
S20	112	S20Ex1F	ACGAWCAAGTCGGTCAGGAA	64.6	S20Ex2R	GGAGCYTTRCCAGTGTCTTT	57.4
S20	113	S20Ex2F	AGCCGYAAYGTCAAGTCTCT	57.9	S20Ex3R	CTCCTTRGCACCWCGGATCA	65.1
S20	114	S20Ex3F	CTGTGCGYATGCCYACCAAG	63.2	S20Ex4R	GTGATCTGCTTRACRATCTC	53.7
S21	115	S21Ex2F	GARTTCGTGGACCTGTACGT	61.4	S21Ex3R	ATRGAGGCRTGGTCCTTGGC	65.1
S21	116	S21Ex3F	GACCAYGCCTCYATCCAGAT	60.5	S21Ex4R	GTCTTGAACTGKCCATTRAA	54.1
S21	117	S21Ex4F	TTCAAGACCTAYGCYATCTG	54.4	S21Ex5R	GMCACRATGCTGTCGGTCTT	62.8
S24†	118	S24Ex2F	TGCTTCAGAGGAAGCAAATG	63.0	S24Ex3R	ATGTACAARACCACCCCTGA	61.6
S24†	119	S24Ex3F	GCMAAGAARAATGAGCCCAA	63.8	S24Ex4R	TTCATTCTGTTCTTGCGTTC	60.4
S25†	120	S25ExAF	AARKCCAAAAAGGACAAGGA	59.9	S25ExBR	TCGAAGAGGACCAGGTTGTT	63.6
S25†	121	S25ExBF	ATCCGDGGCTCYCTGGCCAG	70.1	S25ExCR	TTTYGACACCARTTTGATCA	58.9
S26	122	S26Ex2F	ATCAGGAAYATWGTGGAGGC	57.1	S26Ex3R	ACACAGTARTGYAGCTTCAC	51.5
S28	123	S28Ex1F	GATGCCAGYCGYGTGCAGCC	71.7	S28Ex2R	CCCTGRGARCCAGTTCTTCC	62.6
S28	124	S28Ex2F	GGAAGAACTGGYTCYCAGGG	62.6	S28Ex3R	ATGATKGAKCGGTTGCTGTC	62.5
S29	125	S29Ex1F	CAGCAGCTCTAYTGGAGYCA	60.7	S29Ex2R	CACTGRCGGCACATRTTGAG	61.8
S30	126	S30Ex2F	CACACCCTTGAGGTGACSGG	70.1	S30Ex3R	CCCTCYAGATYCTGGACATG	58.6
S30	127	S30Ex3F	TCAGAGYACTGCACYCTGGA	58.7	S30Ex4R	ACTTTTCCRGCACGRGCCAG	66.6
S30	128	S30Ex4F	CTGGCYCGTGCYGGAAAAGT	66.6	S30Ex5R	TTGGCRCGRCCAGTCTTCTT	64.7

† Genes found in the draft genome sequences of the Pacific bluefin tuna (*Thunnus orientalis*)

* Selected primer pairs to investigate universal specificity

found in the draft genome sequences of the Pacific bluefin tuna. Twelve primer pairs (Table 1, asterisk) expected to amplify 500–1000 bp fragments in the Pacific bluefin tuna were selected, and the PCR amplification results are shown in Fig. 1 and summarized in Table 2. All amplified fragments were larger than those expected for intronless amplicons when separated using agarose gel electrophoresis. Amplified fragments were observed for all primer pairs in the Japanese puffer (lane 2), starry flounder (lane 3), and the Pacific bluefin tuna (lane 4), whereas no amplification was observed for primer pair 17 in the Japanese eel (lane 6), for primer pair 86 in broadbanded thornyhead (lane 5), and for three primer pairs (17, 68, and 86) in the Japanese pilchard (lane 1). The Orders to which the Japanese eel, broadbanded thornyhead, and the Japanese pilchard belonged were not involved in primer design, which may cause the slight difference in amplification success between the former and later fish groups. The highest score for single fragment amplification was observed in starry flounder (10 of 12 primer pairs) and the lowest was observed in the Pacific bluefin tuna and broadbanded thornyhead (6 of 12 primer pairs), whereas the total score for single fragment amplification was relatively high (62.5 %) (45 of 72 primer pairs). Clear or nearly two-band PCR products were observed for primer pair 26 in Japanese pilchard and Pacific bluefin tuna, for primer pair 38 in Japanese eel, for primer pair 49 in starry

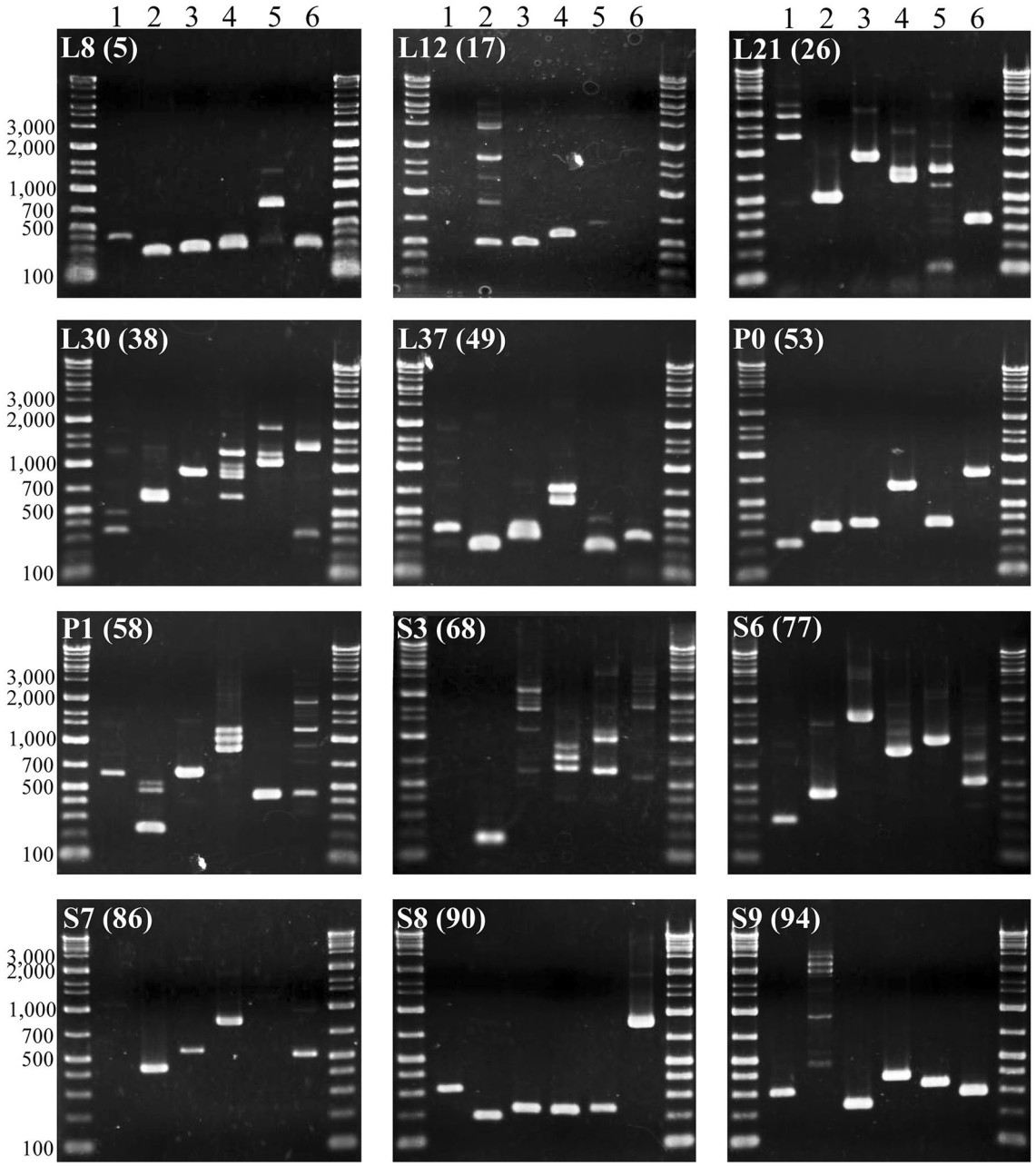

Fig. 1 Agarose gel electrophoresis images of the PCR products amplified using 12 selected primer pairs for ribosomal protein gene (RP) introns. RP genes are shown at the *top-left* in each gel image and the *primer pair number* is shown in *parenthesis* (see also Table 1). The *left* and *right* most ends in each gel are the size marker, and the 1st to 6th lanes are the Japanese pilchard, the Japanese puffer, starry flounder, the Pacific bluefin tuna, broadbanded thornyhead, and the Japanese eel, respectively

flounder and Pacific bluefin tuna, and for primer pair 68 in Pacific bluefin tuna and broadbanded thornyhead. These could be heterozygotes and potential candidates for population genetic analysis. Amplification of three or more fragments was relatively fewer incidence (12 of 72 primer pairs), which may be attributable to paralogs, pseudogenes, and/or non-specific annealing. Elevation of annealing temperature may decrease number of amplified fragments as demonstrated previously (Chow et al. 2015). The sizes of amplified fragments of Pacific bluefin bluefin tuna using nine primer pairs (Table 2, dagger) corresponded to those expected from the genomic data, whereas fragments amplified using three primer pairs (5, 90, and 94) were shorter than

Table 2 Number of fragments amplified by 12 primer pairs in six fish species

RP*	PB	IN	EX	Pilchard	Puffer	Flounder	Tuna	Rockfish	Eel
L8 (5)	795	605	190	1	1	1	1	3	1
L12 (17)	573†	433	140	0	5+	1	1	1	0
L21 (26)	661†	566	95	2+	1	1	2	4+	1
L30 (38)	935†	843	92	2	1	1	5+	3	2
L37 (49)	725†	657	68	1	1	2	2	2	1
P0 (53)	658†	548	110	1	1	1	1	1	1
P1 (58)	985†	887	98	1	3	1	3	1	3+
S3 (68)	736†	662	74	0	1	4+	3	3	2+
S6 (77)	857†	741	116	1	1	1	2+	1	2+
S7 (86)	825†	733	92	0	1	1	1	0	1
S8 (90)	693	553	140	1	1	1	1	1	1
S9 (94)	600	522	78	1	5+	1	1	1	1

Pilchard (*Sardinops melanostictus*), puffer (*Takifugu rubripes*), tuna (*Thunnus orientalis*), rockfish (=broadbanded thornyhead) (*Sebastolobus macrochir*), and eel (*Anguilla japonica*)

PB total fragment size expected for Pacific bluefin tuna (*Thunnus orientalis*), *IN* intron size expected for Pacific bluefin tuna, *EX* expected fragment size for intron less amplification

* Target ribosome protein gene and primer pair number (in parenthesis) used

† Size of fragment amplified was corresponding to that expected from genomic data of Pacific bluefin tuna

expected, indicating that these three primer pairs annealed and amplified non-target regions of the Pacific bluefin tuna genome.

Although nucleotide sequence analysis may be necessary to characterize and authenticate the amplicons of interest, the present study offers new sets of nuclear primers that are potentially applicable to wide variety of fish taxa and can be an initial step toward isolating single-copy nuclear DNA sequences. Subsequent polymorphism detection may also provide a more informative database for genetic species identification and population studies.

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