

Universal primers for exon-priming intron-crossing (EPIC) PCR on ribosomal protein genes in marine animals

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Abstract Relatively conserved exon sequences among distant aquatic animal taxa (Copepoda, Echinodermata, and Teleostei) were determined for 12 ribosomal protein genes, and 19 primer pairs were designed for an exon-primed intron-crossing polymerase chain reaction strategy. The universal utility of these primers was evaluated in distant animal species (sea urchin, squid, crab, shark, and tuna). Fragment amplification was confirmed for at least three species for all primer pairs, and single fragment amplification was observed for at least one species in 16 primer pairs. Primer sets presented here may serve as an initial step for isolating single-copy nuclear DNA sequences in a wide variety of marine animals.

Keywords Universal primers · Ribosomal protein genes · Intron · Single-copy nuclear loci · Marine animals

Introduction

Introns may be a good source for finding DNA polymorphisms in the eukaryotic genome as they rapidly accumulate mutations without greatly affecting gene function. Exon-primed intron-crossing (EPIC) polymerase chain reaction (PCR) has been developed and applied to find genetic polymorphisms in a wide variety of animals (Lessa 1992; Palumbi and Baker 1994; Corte-Real et al. 1994; Daguin et al. 2001; Chow and Takeyama 2000; Chow et al. 2007). Primers designed to target conserved areas may have a wide utility across a broad range of animal taxa (Chow and Hazama 1998; Hassan et al. 2002; Chow and Nakadate 2004; Pinho et al. 2010; Jennings and Etter 2011). The universality of the primers for EPIC PCR depends on the conservation of exonic sequences, and the efficiency of PCR amplification may be primarily affected by gene copy number, including pseudogenes. Genes encoding ribosomal proteins (RPs) may be highly suitable for designing universal primers, as RPs are the most highly conserved genes among eukaryotes and are typically encoded by a single gene (Wool 1979; Wool et al. 1995). Indeed, the coding sequences of RP genes are reported to be highly conserved among humans, fruit flies, and nematodes (Yoshihama et al. 2002). In this study, we designed 19 primer pairs in exons of 12 RP genes and demonstrated the universal utility of these primer pairs using samples of distantly related aquatic animal species.

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Materials and methods

Eighty different ribosomal protein genes were mapped to human chromosomes (Uechi et al. 2001), and cDNA data for the homologous genes of the copepod (*Lepeophtheirus salmonis*, *Acartia pacifica*, or *Calanus helgolandicus*), sea urchin (*Strongylocentrotus purpuratus* or *Paracentrotus lividus*), and fish (*Danio rerio* or *Ictalurus punctatus*) were obtained from the GenBank database. RP genes that were not found in all three of these reference animal taxa were not analyzed. BLAST searches (Altschul et al. 1990) using these reference cDNA sequences against the draft genome sequences of the Pacific bluefin tuna (*Thunnus orientalis*; accession

Table 1 Sequences of primers for amplifying 19 introns of 12 ribosomal protein genes

Pair	Gene	Primer	Orientation	Primer sequence (5–3')	Distance ^a
A	S2	RPS2exCF	Forward	CCHGTNCCCAAGAAGCTGCT	70
	S2	RPS2exDR	Reverse	GGTYTCCTTCCASAGRTCAG	43
B	S3	RPS3ex1F	Forward	AAATCTCVAAGAAGAGRAAG	2
	S3	RPS3ex2R	Reverse	ACRCCGGAGTANCCATCCTC	62
C	S3	RPS3ex2F	Forward	GAGGATGGNTACTCCGGYGT	53
	S3	RPS3ex3R	Reverse	CKCTTCTGVACMACAGCGGT	48
D	S3	RPS3ex3F	Forward	ACCGCTGTKGTBCAGAAGMG	30
	S3	RPS3ex4R	Reverse	GTCGCAACCTTCTCAGCRTA	24
E	S3	RPS3ex4F	Forward	TAYGCTGAGAAGGTTGCGAC	74
	S3	RPS3ex5R	Reverse	AASCKCAGRACACCATARCA	6
F	S14	RPS14exAF	Forward	GCCTCMTTYAACGAYACCTT	29
	S14	RPS14exBR	Reverse	CKGTCRGCTTYACCTTCAT	30
G	S17	RPS17exBF	Forward	AGWGGMATCTCCATCAARCT	49
	S17	RPS17exCR	Reverse	GGRTCACCTCGATGATSTC	18
H	S23	RPS23exAF	Forward	TCACACGCCAARGGCATYGT	15
	S23	RPS23exBR	Reverse	ATRGCAAGTTRGGCTGCTT	14
I	S23	RPS23exBF	Forward	AAGAACGGCAARAAGATCAC	42
	S23	RPS23exCR	Reverse	TGACCYTTACGWCCRAATCC	26
J	S27	RPS27exAF	Forward	GCTAARATYCARAYAAAGGA	4
	S27	RPS27exBR	Reverse	GTGCGKCCRTCYTCCAGCTG	43
K	L3	RPL3exAF	Forward	GGCTACAAGGCYGGYATGAC	39
	L3	RPL3exBR	Reverse	CAGTTYTTGTAGAACKCGACG	149
L	L3	RPL3exBF	Forward	GCGTCGTTCTACAARAACCT	2
	L3	RPL3exCR	Reverse	GTGAARGCCTTCTTCTYGGGA	9
M	L3	RPL3exDF	Forward	CAAGGGWCACGGRTDCAAGG	2
	L3	RPL3exER	Reverse	GTCTTRCGGGGMAGCTTCTT	25
N	L8	RPL8exBF	Forward	AGTTCATCTACTGCGGCAAG	37
	L8	RPL8exCR	Reverse	ATGAYGGTDCCYTCAGGCAT	5
O	L9	RPL9exCF	Forward	RAACTTCYTGGGRGAGAAGT	110
	L9	RPL9exDR	Reverse	ACDGTGGTGGCYTGCTGGAT	10
P	L11	RPL11exAF	Forward	CARACGCCHGTCTTCTCCAA	5
	L11	RPL11exBR	Reverse	AAGGATCKYACAGTGTAGCG	4
Q	L11	RPL11exBF	Forward	CGCTACACTGTRMGATCCTT	87
	L11	RPL11exCR	Reverse	CCCARRTCGATGTGYTCCTG	65
R	L17	RPL17exAF	Forward	GAGWCMGCTCAGGCCATCAA	132
	L17	RPL17exBR	Reverse	TCGGCRCTCTTCTTRGGCCA	8
S	L24	RPL24exBF	Forward	AGMCGCAARCACAAGAAGGG	13
	L24	RPL24exCR	Reverse	ACYTCAGGCTTCTGRITCCT	85

^a Number of nucleotides between 3' end of the primers and start or end of the target introns

nos. BADN01000001-BADN01133062 in the DNA Data Bank of Japan) (Nakamura et al. 2013) detected partial sequences of 31 RP genes among 35 bluefin tuna scaffolds. For each of the 31 genes detected, a multiple sequence alignment was constructed by ClustalX version 1.83 (Thompson et al. 1997), and conserved regions suitable for designing universal primers were surveyed. Crude DNA samples of the long-spined sea urchin (*Diadema savignyi*), neon flying squid (*Ommastrephes bartramii*), golden crab (*Chaceon granulatus*),

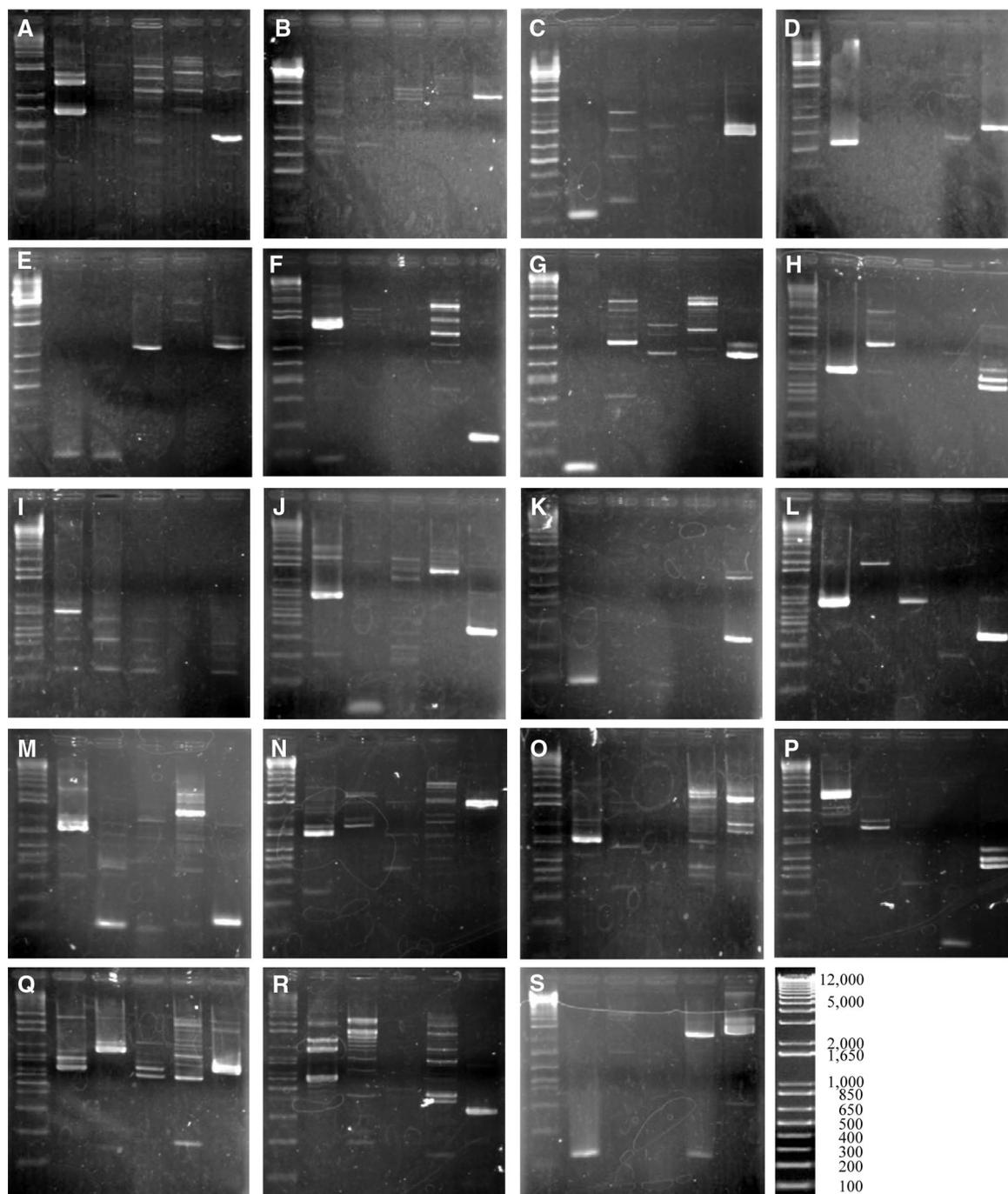


Fig. 1 Agarose gel electrophoresis images of the PCR products obtained by 19 primer pairs using a 55 °C annealing temperature. **a–s** Correspond to the primer pairs shown in Table 1. The *left-most lane (1st lane)* of each gel image is the size marker (1 Kb Plus DNA Ladder, Invitrogen), and the *2nd–6th lanes* correspond to long-spined sea urchin (*Diadema savignyi*), neon flying squid (*Ommastrephes bartramii*), golden crab (*Chaceon granulatus*), mako shark (*Isurus oxyrinchus*), and skipjack tuna (*Katsuwonus pelamis*), respectively



mako shark (*Isurus oxyrinchus*), and skipjack tuna (*Katsuwonus pelamis*) archived in our laboratory were used for evaluating the universal utility of the primers. PCR amplifications were performed in a 12 μ L final volume containing 1 μ L of template DNA (20–50 ng/ μ L), 1.2 μ L 10 \times buffer, 1 mM of each deoxynucleotide phosphate (dNTP), 0.4 μ M of each primer, and 0.5 units of EX Taq polymerase (Takara, Japan). The same reaction conditions were applied for all primer pairs in which the reaction mixtures were preheated to 94 $^{\circ}$ C

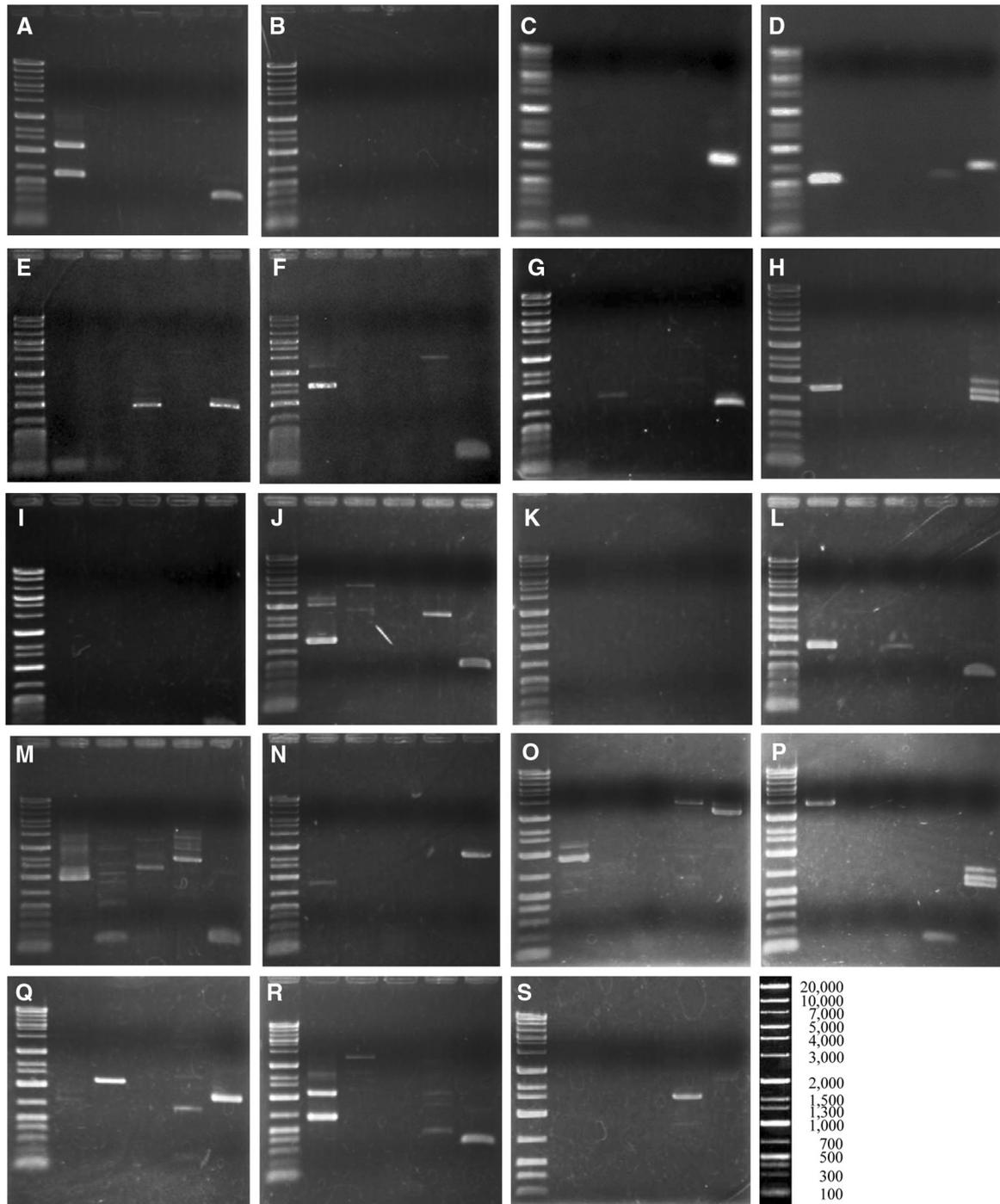


Fig. 2 Agarose gel electrophoresis images of the PCR products obtained by 19 primer pairs at a 58 $^{\circ}$ C annealing temperature. The left-most lane (1st lane) of each gel image is the size marker (Gene Ladder Wide 1, Wako), and the species used are the same as those in Fig. 1

for 4 min, followed by 35 amplification cycles (94 °C for 30 s, 55 or 58 °C for 30 s, and 72 °C for 50 s), with a final extension at 72 °C for 7 min. PCR products were electrophoresed on a 1.5 % agarose gel (UltraPure Agarose, Invitrogen, Carlsbad CA), stained with ethidium bromide and photographed.

Results and discussion

Relatively conserved exon sequences among distant reference animal taxa were determined for 12 genes in which 19 primer pairs (A–S) were designed for EPIC PCR (Table 1). To design the primers, we adopted a primer length of 20 bases, a moderate GC content (approximately 50 %), and usually less than three degenerated bases, and because the conserved regions among the variety of taxa were not extensive, there were limited options for selecting primer positions.

At a lower annealing temperature (55 °C), amplification of single to multiple fragments ranging from approximately 100–4000 bp (fragments smaller than 100 bp were not counted) was observed in at least three taxa for each primer pair (Fig. 1). The number of amplified fragments decreased at the higher annealing temperature (58 °C), to the extent that no amplified fragments were obtained for three primer pairs (Fig. 2b, i, and k). Amplification failure may be attributable to primer mismatch, large numbers of processed pseudogenes (Duvov and Perry 1984), and/or non-specific annealing, which may be responsible for multiple fragment amplification. At the higher annealing temperature, the number of primer pairs which amplified a single fragment was 13 pairs in tuna, followed by 8 in sea urchin, 6 in shark, 3 in squid, and only 2 in crab. Higher scores observed in tuna and sea urchin are reasonable, since RP gene data of teleost and sea urchin were used to design primers. The lowest result in crab may indicate distant relationship between copepod and decapod.

Two to three amplicons (13 in total) from five primer pairs (D, E, G, L, and Q) showing a single fragment amplification at the higher annealing temperature (Fig. 2) were selected and subjected to direct nucleotide sequencing using PCR primers. Partial or complete nucleotide sequences were successfully determined for eight (one each from primer pairs D, E, and G; two from L; and three from Q) out of 13 amplicons. Conserved exon sequences and expected exon–intron motifs for the respective RP genes were confirmed in six amplicons from D, E, G, and Q, whereas these were not determined in two amplicons from L due to the short distance between the primer annealing site and an intron (see Table 1). All sequences are available in DDBJ and GenBank under the accession numbers LC055480–LC055485, LC055565, LC055566.

Although intensive investigation into the optimal PCR conditions was not performed, amplification failure and multiple fragment amplification may be improved by controlling the annealing temperature or modifying the primer sequences for taxa of interest.

Thus, the present study offers new nuclear primers potentially applicable to a wide variety of marine animal taxa, which may be an initial step toward isolating single-copy nuclear DNA sequences in a wide variety of marine animals. Subsequent polymorphism detection may also provide a more informative database for genetic species identification and population study.

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