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ORIGINAL RESEARCH

Locus-specific EPIC-PCR primers for four distinct calmodulin genes of the Pacific bluefin tuna *Thunnus orientalis* (Temminck & Schlegel, 1844)

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Abstract Nucleotide sequences of four distinct calmodulin genes (designated as *CaM*-A to -D) of the Pacific bluefin tuna (*Thunnus orientalis*) were compared. Nucleotide sequence divergence between the coding regions of four genes ranged from 16.0 to 21.6%, and locus-specific eight primer pairs targeting introns were designed. These primer pairs amplified expected size of fragments in five congeneric tuna species, and utility of these primer pairs was investigated using distant fish species.

Keywords *Thunnus orientalis* · Four calmodulin genes · Locus-specific primers · Cross-species amplification · DNA marker

Introduction

The Pacific bluefin tuna (*Thunnus orientalis*) is one of the largest bony fish and most commercially valuable species. Because of the overexploitation and the declining stock status, this species has been listed as vulnerable by the IUCN. To date, polymorphic DNA markers developed and used for the Pacific bluefin tuna are microsatellite and mitochondrial DNA (Takagi et al. 1999; Nomura et al. 2014; Yagishita et al. 2014).

Introns rapidly accumulate mutations, which may be used as selectively neutral nuclear genetic marker complementing microsatellite and mitochondrial DNAs. Exon-primed intron-crossing (EPIC) PCR strategy employs conserved exon sequences to design universal primers (Lessa 1992). The efficiency of isolating a single copy of the target nuclear genome may be dependent on the number of paralogs and pseudogenes. Targeting highly conserved single copy of nuclear gene such as ribosomal protein gene has been successful to design universal primers applicable to wide variety of marine animals (Chow and Hazama 1998; Chow and Yanagimoto 2016). Calmodulin (CaM) is one of the most highly conserved molecules. Two to four distinct genes have been detected in vertebrates (Chien and Dawid 1984; Simmen et al. 1985; Nojima and Sokabe 1987; SenGupta et al. 1987; Nojima 1989; Koller et al. 1990; Matsuo et al. 1992; Rhyner et al. 1994; Friedberg and Rhoads 2002), and three to four distinct CaM-encoding cDNAs were reported in fish (Matsuo et al. 1992; Friedberg and Rhoads 2002). Despite considerable amount of nucleotide substitutions between

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distinct *CaM* genes, amino acid sequences of the metazoan CaMs are extremely conserved (Yuasa et al. 1999, 2001).

The draft genome sequence of the Pacific bluefin tuna has been reported (Nakamura et al. 2013), in which we detected four distinct *CaM* genes. In this study, we compared whole structure of these four tuna *CaM* genes and designed locus-specific primers for EPIC-PCR. We also investigated the utility of these primer pairs in other fish species.

Materials and methods

Fish samples used in this study were derived from our laboratory stock collected by scientific sampling. Matsuo et al. (1992) reported cDNAs of four *CaM* genes (*CaM*-A to -D) in medaka *Oryzias latipes* (DDBJ/EMBL/GenBank accession numbers D10363 to D10366). We used these sequences as in silico probes to detect similar sequences in the draft genome of the Pacific bluefin tuna (http://nrifs.fra.affrc.go.jp/ResearchCenter/5_BB/genomes/Tuna_DNAmicroarray/index.html). BLAST searches (Altshul et al. 1990) using these probes were performed, and we detected four scaffolds (Ba00000990, Ba00001092, Ba00001784, and Ba00003491) and confirmed these to contain whole *CaM* genes. *CaM* gene sequence in one scaffold (Ba00000990) was complete, while partial intron sequences were not determined in the other three scaffolds (Ba00001092, Ba00001784 and Ba00003491). We designed oligo nucleotide primers at flanking regions of these undetermined sequences for PCR. PCR mixtures were preheated at 94 °C for 4 min, followed by 35 amplification cycles (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 50 s), with a final extension at 72 °C for 7 min. PCR products were cloned using TOPO TA Cloning Kit (Invitrogen). Transformed colonies were subjected to colony direct PCR using M13 forward and M13 reverse primers, and PCR products were purified via ExoSAP-IT (GE Healthcare Life Sciences). Nucleotide sequencing reaction was performed using the BigDye Terminator Cycle Sequencing Kit Ver3.1 (Applied Biosystems) with the PCR primers and M13 forward and M13 reverse primers, and the nucleotide sequences were determined with an automated DNA sequencer (ABI PRISM 3730XL Genetic Analyzer) (Applied Biosystems).

Exon sequences of medaka and Pacific bluefin tuna determined were subjected to BLAST search in GenBank to find similar sequences of teleostei. Among sequences shown by BLATS search, those from distant fish taxa but common to medaka and Pacific bluefin tuna were selected. Three species and the sequences selected were barramundi *Lates calcarifer* (accession no. XM_018694615, XM_018694749, XM_018695169, XM_018702063), mummichog *Fundulus heteroclitus* (XM_012861547, XM_012862386, XM_012868907, XM_012877456), and mangrove killifish *Kryptolebias marmoratus* (XM_017404200, XM_017409196, XM_017424744, XM_017426177). Barramundi belongs to Perciformes, while mummichog and mangrove killifish belong to Cyprinodontiformes.

Alignment of exon sequences was performed using ClustalW algorithm implemented in MEGA6 (Tamura et al. 2013), and K2 + G + I was selected as the best fit model. Kimura 2-parameter distance (K2P) between the exon sequences was calculated and phylogenetic tree was constructed using MEGA6.

Results

Nucleotide sequences of the four tuna *CaM* genes determined in the present study are available in DDBJ/EMBL/GenBank (LC183900 to LC183903). All these *CaM* genes are consisted of six exons and five introns. Exon sequences of these four genes were identical in length (450 bp), and K2P distances between the exon sequences ranged from 16.0 to 21.6%. All nucleotide substitutions among the sequences were silent. Phylogenetic tree constructed using exon sequence data of medaka, Pacific bluefin tuna, barramundi, mummichog and mangrove killifish is shown in Fig. 1. Four large clades appeared in the tree, and mean K2P ± S.E. within and between these clades ranged from 5.8 ± 0.9 to 13.4 ± 1.7% and from 16.7 ± 2.2 to 23.5 ± 3.0%, respectively. Four tuna *CaM* genes were designated as A to D according to the affinity with medaka *CaM* genes. Structures of four tuna *CaM* genes (A to D) are shown in Fig. 2, and introns of 300 to 1500 bp long were selected for EPIC-PCR (closed areas of horizontal bars). As the 1st exon was three bases long for all genes, the 1st introns were not included for the target. We designed eight locus-specific oligo-primer (20 nt)

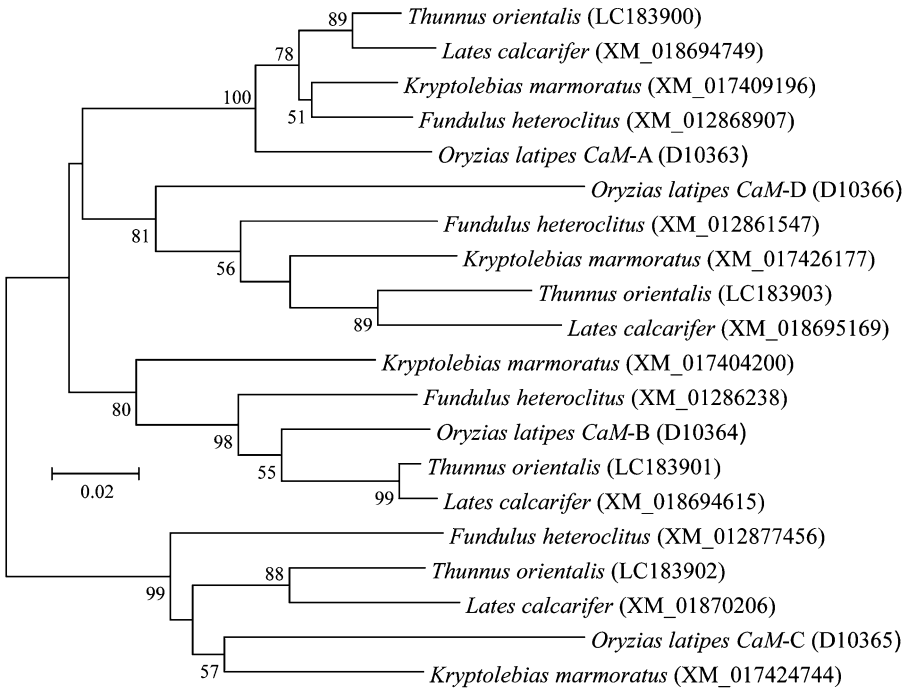

Fig. 1 Unrooted neighbor-joining phylogenetic tree (K2 + G + I) using exon sequences of distinct *CaM* genes of the Pacific bluefin tuna (*Thunnus orientalis*), medaka (*Oryzias latipes*), barramundi (*Lates calcarifer*), mummichog (*Fundulus heteroclitus*), and mangrove killifish (*Kryptolebias marmoratus*). Bootstrap values >50% (out of 1000 replicates) are shown at the nodes. Accession *numbers* are shown in the *parenthesis*. Essentially the same tree topology was obtained in maximum likelihood and maximum parsimony trees

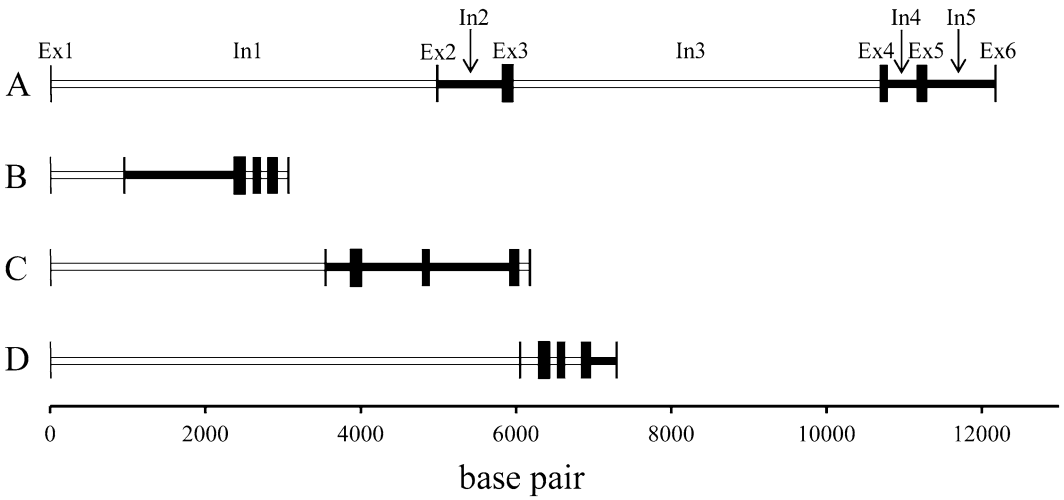

Fig. 2 Genomic structures of four *CaM* genes (A to D) of the Pacific bluefin tuna. *Vertical bars* represent exons (Ex), and *horizontal bars* are introns (In). Introns of 300 to 1500 bp long (*black horizontal bars*) are chosen as targets for exon-primed intron-crossing PCR

pairs based on the nucleotide substitutions between exon sequences of the Pacific bluefin tuna (Fig. 3, underlined), using which fish species shown in Table 1 were subjected to PCR amplification under above-mentioned condition. Since the size of amplified fragments in all *Thunnuns* spp. used was the same, the Pacific bluefin tuna (2nd lane) was shown as a representative for all *Thunnus* spp. In tuna, a single fragment amplification was observed with seven primer pairs (Fig. 4a–c, e–h) and two fragments were observed in one primer pair (Fig. 4d), in which fragment size amplified by seven primer pairs (Fig. 4a–c, e–h) and longer

	ex2F	2nd intron	ex3R	
CaM-A	GCTGACCAACTAACAGAGGA[11nt]	~	[12nt]CTTTCTCCTTATTCGACAAG	882†
CaM-B	GCTGATCAGCTTACAGAAGA[11nt]	~	[12nt]CATTTTCGCTCTTTGACAAG	1464
CaM-C	GCTGATCAGCTGACTGAAGA[11nt]	~	[12nt]CATTCTCGCTGTTTGACAAG	369
CaM-D	GCTGACCAACTAACAGAAGA[11nt]	~	[12nt]CTTTCTCCCTGTTTGATAAG	281
	***** ** ** ** ** **		* ** ** * ** ** ***	
	ex3F	3rd intron	ex4R	
CaM-A	AGCTTGGCACCGTCATGAGG[64nt]	~	[7nt]AACCATTGACTTCCCGGAGT	4835
CaM-B	AGCTGGGCACAGTCATGCGT[64nt]	~	[7nt]AACGATAGACTTCCCGGAGT	206
CaM-C	AGCTTGGGACTGTGATGCGC[64nt]	~	[7nt]TACAATTGATTTTCCTGAGT	886
CaM-D	AGCTGGGTACAGTCATGAGG[64nt]	~	[7nt]CACCATCGACTTCCCTGAGT	206
	**** ** ** ** *** *		** ** ** ** ** ****	
	ex4F	4th intron	ex5R	
CaM-A	GGAGATCCGCGAGGCTTTCC[14nt]	~	[64nt]AACAGACGAGGAGGTGGACG	490
CaM-B	GGAGATCAGAGAAGCATTCC[14nt]	~	[64nt]GACTGATGAAGAAGTGGATG	196
CaM-C	GGAGATCAGAGAAGCCTTCA[14nt]	~	[64nt]CACTGATGAAGAGGTGGACG	1135
CaM-D	AGAGATCAGAGAGGCATTCA[14nt]	~	[64nt]GACAGACGAGGAAGTAGACG	317
	****** * ** ** ***		** ** ** ** ** ** *	
	ex5F	5th intron	ex6R	
CaM-A	AGAAGCTAACAGACGAGGAG[45nt]	~	[17nt]TGTACAGATGATGACTGCAA	968
CaM-B	AGAAGCTGACTGATGAAGAA[45nt]	~	[17nt]CGTACAAATGATGACGGCGA	225
CaM-C	AGAAGCTCACTGATGAAGAG[45nt]	~	[17nt]CGTCCAGATGATGACTGCCA	223
CaM-D	AGAAGCTGACAGACGAGGAA[45nt]	~	[17nt]CGTTCAGATGATGACCGCCA	416
	******* ** ** ** **		** ** ******** ** *	

Fig. 3 Alignments of partial exon sequences of four *CaM* genes (A to D) of the Pacific bluefin tuna, flanking 2nd to 5th introns. Introns are shown by tilde, and number of nucleotides between primer and intron is presented in *bracket*. Identical nucleotides among genes (A to D) are indicated by *asterisk*, and nucleotide sequences adopted for designing primers are *underlined*. †Predicted size (bp) of amplified fragment for the Pacific bluefin tuna

Table 1 Bony fish species used in this study

Species	Common name	Order	Catch locality (n: sample size)
Thunnus orientalis	Pacific bluefin tuna	Perciformes	Northwest Pacific (3)
Thunnus thynnus	Atlantic bluefin tuna	Perciformes	Northwest Atlantic (3)
Thunnus maccoyi	Southern bluefin tuna	Perciformes	Southwest Indian Ocean (2)
Thunnus alalunga	Albacore	Perciformes	Northwest Pacific (2)
Thunnus albacares	Yellowfin tuna	Perciformes	Northwest Pacific (2)
Sardinops melanostictus	Japanese pilchard	Clupeiformes	Tosa Bay, Japan (1)
Takifugu rubripes	Japanese puffer	Tetraodontiformes	Japan Sea (1)
Platichthys stellatus	Starry flounder	Pleuronectiformes	Shibetsu, Japan (1)
Sebastolobus macrochir	Broadbanded thornyhead	Scorpaeniformes	Northwest Pacific (1)
Anguilla japonica	Japanese eel	Anguilliformes	Tone River, Japan (1)

fragment by one primer pair (Fig. 4d) matched well with those predicted from the sequence data (see Fig. 3). We cloned and sequenced the shorter fragment (c.a. 400 bp) amplified by *CaM*-C: ex3F × ex4R (Fig. 4d). This fragment (376 bp, accession no. LC157866) had a same primer sequence (Fig. 3, *CaM*-C: ex3F) at both 5′ and 3′ ends and no *CaM*-like sequence was observed, indicating non-specific amplification.

Amplification efficiency varied among the other fish species. No amplicon was observed in four primer pairs (*CaM*-B: ex2F × ex3R, *CaM*-C: ex2F × ex3R, *CaM*-C: ex3F × ex4R, *CaM*-C: ex4F × ex5R) (Fig. 3) for the Japanese pilchard (3rd lane of Fig. 4b–d, f) and one primer pair (*CaM*-B: ex2F × ex3R) for the Japanese eel (7th lane of Fig. 4b), while amplicons were obtained in all primer pairs in the other species. Single fragment amplification was obtained in 21 out of 40 occasions (5 species by 8 primer pairs).

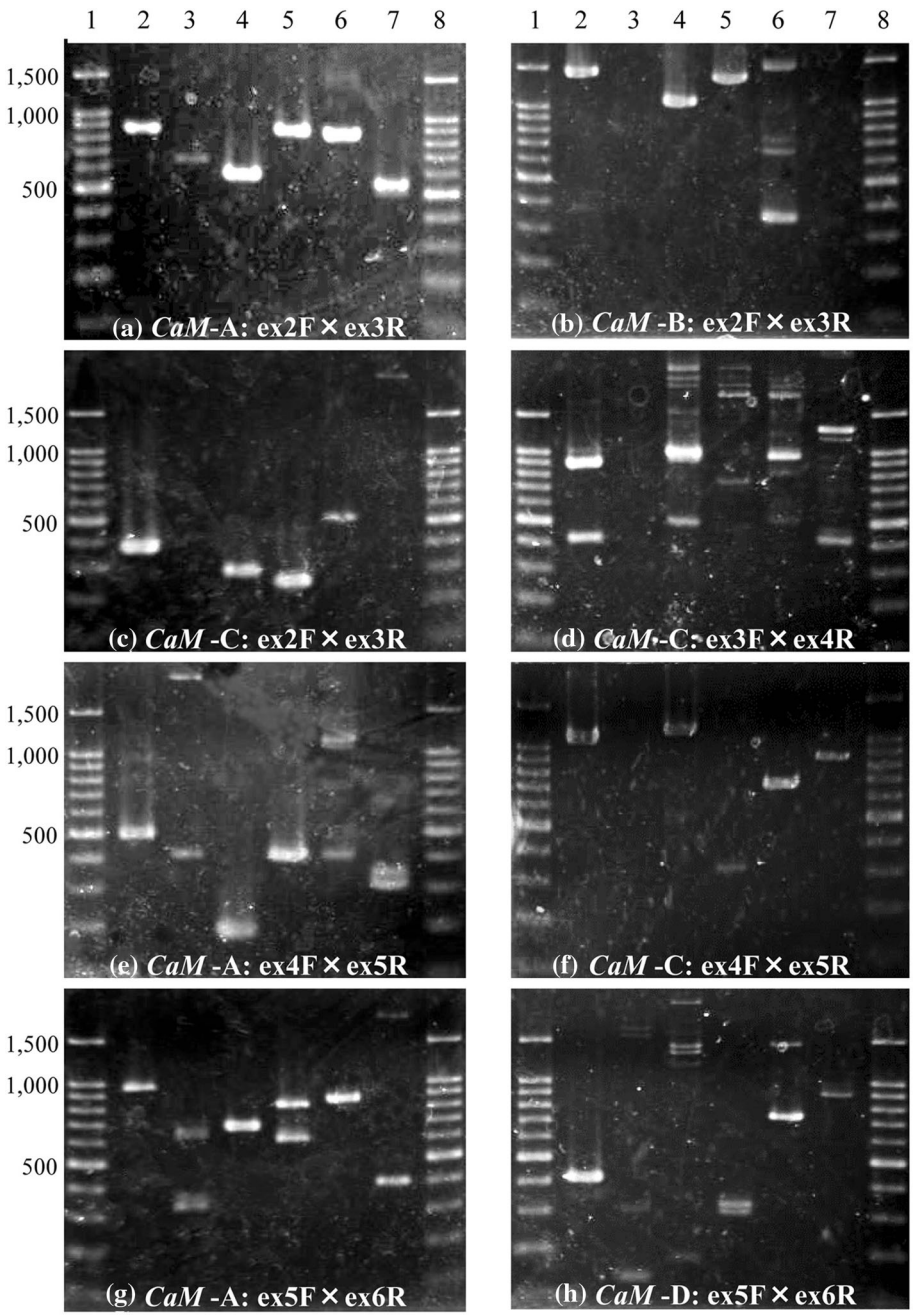

Fig. 4 Agarose gel electrophoresis images of PCR products amplified by eight primer pairs (see Fig. 3). 1st and 8th *lanes* are molecular marker, and the size (bp) is presented at the *left* margin. 2nd to 7th *lanes* are the Pacific bluefin tuna, Japanese pilchard, Japanese puffer, starry flounder, broadbanded thornyhead, and Japanese eel, respectively (see Table 1)

Discussion

One or two CaM-encoding genes have been reported in invertebrates (Hardy et al. 1988; Doyle et al. 1990; Swanson et al. 1990; Côrte-Real et al. 1994; Karabinos and Riemer 1997; Yuasa et al. 1999, 2001). Using locus-specific primers, Côrte-Real et al. (1994) successfully isolated one (*CaM-1*) of two *CaM* genes of mussel *Mytilus edulis*, and length polymorphism found in the 3rd intron was used for population genetic analysis. A high restriction fragment length polymorphism (RFLP) was observed in the *CaM* intron of the Atlantic bluefin tuna *Thunnus thynnus* (Nakadate and Chow 2008). Single-nucleotide polymorphism (SNP) found in the 4th

intron of swordfish *Xiphias gladius* was used to investigate population structure (Chow and Takeyama 2000; Chow et al. 2007). Partial exon sequence (200 bp) of the Atlantic bluefin tuna was observed to have higher homology (99% homologous) with that of *CaM*-A in *T. orientalis* than with the others (*CaM*-B, -C and -D) (83–86%). Similarity of exon sequence (105 bp) of the swordfish *CaM* to medaka *CaM*-A was suggested previously (Chow and Takeyama 2000). *CaM*-A of *T. orientalis* showed much higher homology with the swordfish exon sequence (94% homologous), while *CaM*-B to D of *T. orientalis* were much less homologous (83–86%) with the swordfish exon sequence. *CaM*s of the Atlantic bluefin tuna and swordfish were 94% homologous. These indicate that the primer pair used in these previous studies exclusively amplified homologous intron of *CaM*-A gene. Locus-specific primer pairs presented in this study are designed based on genome data of the Pacific bluefin tuna. Nevertheless, success rate of amplifying single fragment among distant fish species was relatively high, indicating the possibility to develop a genetic assay to target a specific locus in wide variety of fish taxa.

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