

Characterization of two parvalbumin genes and their association with growth traits in Asian seabass (*Lates calcarifer*)

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Summary

Parvalbumins are extremely abundant in fish muscle and play an important role in muscle relaxation. In this study, two parvalbumin genes (*PVALB1* and *PVALB2*) were cloned from Asian seabass (*Lates calcarifer*). The cDNAs for *PVALB1* and *PVALB2* were 840 and 667 bp respectively. Both genes consisted of five exons and four introns, encoded 109 amino acids, and were of beta lineage. Using real-time polymerase chain reaction, expression of *PVALB1* was detected in all 10 tissues tested, with expression in brain, kidney, muscle and small intestine being 15- to 322-fold higher than in the other tissues. Expression of *PVALB2* was detected only in muscle, brain and intestine, and was up to 10-fold lower than *PVALB1* expression. A (CT)₁₇ microsatellite in the 3'-untranslated region of *PVALB1* and three single nucleotide polymorphisms (SNPs) in the third intron of *PVALB2* were identified. The microsatellite in *PVALB1* was significantly associated with body weight and body length at 90 days post-hatch ($P < 0.01$), whereas the SNPs in *PVALB2* were not associated with these traits.

Keywords association, cDNA, parvalbumin, polymorphism, real-time polymerase chain reaction.

The parvalbumin protein family can be subdivided into alpha- and beta-evolutionary lineages (Goodman & Pechere 1977). In mammals, the alpha- and beta-parvalbumin genes consist of five exons and four introns (Fohr *et al.* 1993). It has been suggested that parvalbumins are important in the relaxation of muscle fibres (Muntener *et al.* 1995). Parvalbumins are extremely abundant in fish muscle. Although cDNA sequences of parvalbumin genes have been isolated in some fish species (Lindstrom *et al.* 1996; Swoboda *et al.* 2002), their function and genomic structure in teleosts have not yet been described.

Asian seabass (*Lates calcarifer*) is a marine fish species used for food in Asia and Australia. However, few functional genes have been cloned and characterized (Candy & Collet 2005). No association studies of traits and genes have been conducted in Asian seabass. In this paper, we report the cDNA sequence, expression, genomic organization, polymorphism and association analysis of two parvalbumin genes in Asian seabass.

Total RNA was isolated from 10 tissues (brain, eye, gill, heart, small intestine, kidney, liver, muscle, skin and spleen) of a 3-month-old Asian seabass. Three micrograms of total RNA from each tissue was reverse-transcribed to cDNA with Superscript II (GibcoBRL, Carlsbad, CA, USA), the SMART Oligo II A primer and the CDS primer (Table S1). Normalized cDNA libraries for six tissues (brain, muscle, spleen, kidney, heart and liver) were constructed using a cDNA normalization kit (Evrogen, Moscow, Russia). Of the 8000–10 000 clones picked from each library, inserts of 800 clones were sequenced using the BigDye version 3.0 kit (Applied Biosystems, Foster City, CA, USA) as described (Yue *et al.* 2004). Two clones containing fragments of different parvalbumin genes were isolated from the muscle cDNA library by sequencing clones and comparing known sequences of parvalbumin genes from GenBank (e.g. common carp, AJ292211 and AJ292212). Full-length cDNAs of the two genes were obtained by RACE using the 5'/3'-RACE kit (Roche, Mannheim, Germany) and specific primers PVALB01 and PVALB02 (for *PVALB1*) or PVALB03 and PVALB04 (for *PVALB2*; Table S1). Full-length sequences of *PVALB1* and *PVALB2*, which were 840 and 667 bp, respectively, were deposited into GenBank with accession numbers AY688372 and AY688373 respectively. The open reading frames of both genes were 330-bp long and encoded 109 amino acids. The nucleotide sequences of the coding regions of the two

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genes showed 72.8% similarity. A (CT)₁₇ microsatellite was identified in the 3'-untranslated region (UTR) of *PVALB1*. Phylogenetic analysis of all known fish parvalbumin genes in GenBank showed that the two parvalbumin genes of seabass were of the beta type (data not shown).

Expression of *PVALB1* and *PVALB2* in 10 tissues of a 3-month-old Asian seabass was detected with real-time polymerase chain reaction (PCR) in an ICycler thermal cycler (Bio-Rad, Hercules, CA, USA) by using primers PVALB12 and PVALB13, as well as PVALB04 and PVALB14 (Table S1). The expression of these genes was compared with the expression of the *tubulin, alpha 1* (*TUBA1*) gene detected with primers Lca tub-F and Lca tub-R (Table S1). *PVALB1* was detected in all tissues; however, the expression levels in tissues varied markedly. The highest expression was seen in brain (*PVALB1/TUBA1* = 492) followed by muscle (174), kidney (47), small intestine (24), skin (13), spleen (11), liver (8.3), heart (4), gill (2) and eye (1.5). *PVALB2* was expressed only in muscle (*PVALB2/TUBA1* = 73), brain (40) and intestine (22). It seems that the expression patterns of the two Asian seabass parvalbumin genes differ from those in mammals (Fohr *et al.* 1993), in which parvalbumin genes are expressed mainly in fast-contracting/relaxing muscles but not in intestine.

The complete genomic region of the two parvalbumin genes was amplified with the Long Distance PCR kit (Roche) using primers located in the 5'- and 3'-UTRs of their cDNAs (PVALB05 and PVALB06 for the *PVALB1* gene, and PVALB07 and PVALB08 for the *PVALB2* gene; Table S1). Sequences in the 5'-flanking region were extended using a genome walking kit (BD Clontech, Mountain View, CA, USA) and gene-specific primers (PVALB09 and PVALB10 for the *PVALB1* gene, and PVALB04 and PVALB11 for the *PVALB2* gene; Table S1). The genomic fragments of *PVALB1* (AY688374) and *PVALB2* (AY688375) were 2749 and 1941 bp respectively (Fig. 1). Comparison between genomic DNA and cDNA sequences indicated that both genes were made up of five exons (Fig. 1), similar to the parvalbumin genes in human and mouse (Schleef *et al.* 1992; Fohr *et al.* 1993). A feature of the genomic structure of the two beta-parvalbumins is that the 5'-UTR is split into two exonic fragments (Fig. 1).

The (CT)₁₇ microsatellite in the 3'-UTR of *PVALB1* was genotyped in 22 unrelated individuals collected from a local

farm using primers PVALB06 and PVALB15 (Table S1) as described previously (Yue *et al.* 2004). Two alleles (261 and 263 bp) were detected, and the observed and expected heterozygosities were 0.45 and 0.46 respectively. In the cDNAs of parvalbumin genes in salmon (Lindstrom *et al.* 1996) and common carp (Swoboda *et al.* 2002), there is no microsatellite. In *PVALB2*, three single nucleotide polymorphisms (SNPs) were identified in the third intron by amplifying and sequencing DNA from the 22 individuals using primers Par2-SNP-F and Par2-SNP-R (Table S1). One SNP was located 380 bp downstream of the ATG start codon, and was an A>G substitution (allele frequency 0.36/0.64). Another SNP (A>T) was located at +454 bp (allele frequency 0.77/0.23), and the third was a T>C substitution located at +528 bp (allele frequency 0.41/0.59).

Two adult Asian seabass selected from commercial brooders were paired. Two thousand of their offspring were cultured in a 1-ton cylindrical tank in static conditions following standard culture protocols (Pillay 1990) until 30 days post-hatch (dph), and 400 individuals were transferred to a 3-ton cylindrical tank with continuous seawater flow-through. Juveniles were raised communally in the 3-ton tanks and maintained on strict feeding regimes until 90 dph. Body weights (BW) and body lengths (BL) of 96 randomly selected F1 individuals were measured at 90 dph. Fulton's condition factor K was calculated based on BW and BL. DNA was extracted from fin clips of each fish using a simple method (Yue & Orban 2005). The *PVALB1* microsatellite and the three *PVALB2* SNPs were genotyped. Mendelian inheritance of the polymorphisms was observed. The single-marker linear model was used to examine the relationship between the genotypes of each marker and the growth-related traits using the GLM program of SAS (SAS Inc., Cary, NC, USA). Because all 96 fish came from a single-parent pair and were raised under the same conditions, the model only included the effect of genotype. Duncan's multiple-range test showed that the BW and BL of individuals with the *PVALB1* 263/263 genotype were significantly greater than individuals with genotypes of 261/261 or 261/263 (Table 1). No significant associations were detected between the three *PVALB2* SNPs and the growth traits; furthermore, no associations were found between the *PVALB2* haplotypes and the growth traits (Table 1).

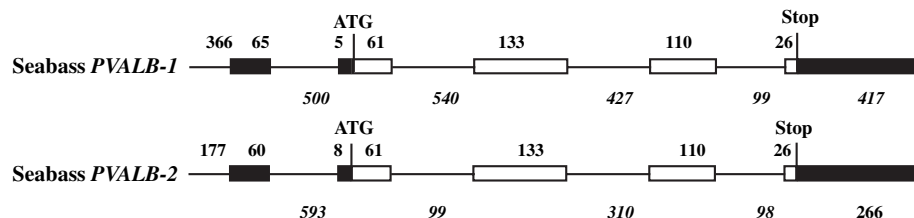


Figure 1 Genomic organization of two beta-parvalbumin genes in Asian seabass (*Lates calcarifer*). White boxes, exons encoding amino acids; black boxes, 5'-untranslated region (UTR) and 3'-UTR; black lines, introns. Numbers represent lengths of each region in base pairs.

Table 1 Growth-related traits of Asian seabass with different genotypes at a microsatellite locus in *PVALB1* and at three single nucleotide polymorphisms (SNPs) in *PVALB2*¹.

		Traits ²		
Genotypes	<i>n</i>	BW (g)	BL (mm)	K
Microsatellites				
261/261	18	26.6 ± 3.2 ^b	96.9 ± 3.8 ^b	2.73 ± 0.03 ^a
261/263	57	20.9 ± 1.0 ^b	89.0 ± 2.2 ^b	2.72 ± 0.04 ^a
263/263	21	38.0 ± 3.5 ^a	110.1 ± 3.4 ^a	2.73 ± 0.03 ^a
SNP (A>G)				
AA	24	24.6 ± 3.7 ^a	93.4 ± 4.2 ^a	2.65 ± 0.03 ^a
AG	55	25.8 ± 1.7 ^a	95.0 ± 2.4 ^a	2.75 ± 0.07 ^a
GG	17	27.0 ± 3.0 ^a	97.4 ± 4.0 ^a	2.73 ± 0.02 ^a
SNP (A>T)				
AA	55	26.5 ± 2.9 ^a	96.2 ± 2.5 ^a	2.70 ± 0.04 ^a
AT	41	24.7 ± 2.03 ^a	93.6 ± 3.2 ^a	2.75 ± 0.03 ^a
SNP (C>T)				
CC	17	27.0 ± 3.0 ^a	97.4 ± 4.0 ^a	2.73 ± 0.02 ^a
CT	55	25.8 ± 1.7 ^a	95.0 ± 2.4 ^a	2.75 ± 0.07 ^a
TT	24	24.6 ± 3.7 ^a	93.4 ± 4.2 ^a	2.65 ± 0.03 ^a
Haplotype combinations				
GAC/GTC	19	28.1 ± 2.8 ^a	98.9 ± 3.7 ^a	2.72 ± 0.03 ^a
GAC/AAT	32	26.7 ± 2.6 ^a	96.4 ± 3.2 ^a	2.74 ± 0.03 ^a
AAT/GTC	21	23.5 ± 3.9 ^a	91.1 ± 4.3 ^a	2.78 ± 0.03 ^a
AAT/AAT	24	24.6 ± 3.0 ^a	93.5 ± 3.7 ^a	2.65 ± 0.07 ^a

¹Significant differences ($P < 0.01$) are shown using different superscripts (a vs. b) across the columns.

²BW, body weight at 90 days post-hatch (dph); BL, body length at 90 dph; K, Fulton's condition factor.

In conclusion, we cloned cDNAs of two beta-parvalbumin genes in Asian seabass, determined their genomic structure, examined their expressions in 10 tissues, and revealed a significant association between a *PVALB1* polymorphism and growth-related traits. This information could facilitate further understanding of the functions and regulation of the two parvalbumin genes in fish growth.

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Supplementary Material

The following supplementary material is available for this article online from <http://www.blackwell-synergy.com>:
Table S1 Primers used in PCR, RT-PCR and real-time PCR reactions for the Asian seabass *PVALB1* and *PVALB2* genes.