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Molecular and comparative analyses of type IV antifreeze proteins (AFPIVs) from two Antarctic fishes, *Pleuragramma antarcticum* and *Notothenia coriiceps*

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ABSTRACT

Antifreeze protein type IV (AFPIV) cDNAs and genomic DNAs from the Antarctic fishes *Pleuragramma antarcticum* (Pa) and *Notothenia coriiceps* (Nc) were cloned and sequenced, respectively. Each cDNA encoded 128 amino acids, with 94% similarity between the two and 83% similarity with AFPIV of the longhorn sculpin, *Myoxocephalus octodecemspinosus*. The genome structures of both genes consisted of four exons and three introns, and were highly conserved in terms of sequences and positions. In contrast, the third intron of PaAFPIV had additional nucleotides with inverted repeats at each end, which appeared to be a MITE-like transposable element. Comparative analysis revealed that fish AFPIVs were widely distributed across teleost fishes, well conserved in their intron positions, but more variable in intron sequences and sizes. However, the intron sequences of two Antarctic fishes were highly conserved, indicating recent radiation of notothenioids in the evolutionary lineage. The recombinant PaAFPIV and NcAFPIV were expressed in *E. coli*, and examined antifreeze activity. PaAFPIV and NcAFPIV gave ice crystals with star-shaped morphology, and thermal hysteresis (TH) values were 0.08 °C at the concentration of 0.5 mg/ml.

1. Introduction

Antarctic fishes are well known to produce active AFGP and/or AFPs to survive ice-laden seawater (DeVries 1971; Hsiao et al., 1990; Wohrmann 1996; Jin and DeVries, 2006). Fish antifreeze proteins (AFPs) are expressed and secreted into the serum to protect fishes living in icy seawater from freezing. They are a structurally diverse group of proteins, consisting of antifreeze glycoproteins (AFGPs) and AFP, types I–IV (Davies and Hew, 1990; Harding et al., 1999; Fletcher et al., 2001; Kristiansen and Zachariassen, 2005). Of these, type IV AFP from the blood of the longhorn sculpin, *Myoxocephalus octodecemspinosus* (Mo), which is an unusual protein with no similarity with other types of fish AFPs, was identified and classified as a new type IV AFP. The three-dimensional structure of it was proposed to be a left-handed, antiparallel, four-helix, bundle model (Deng and Laursen, 1998), and this protein is suggested to have evolved from apolipoproteins (Cheng 1998). Although type IV AFP function is still controversial, it was hypothesized that AFPIVs might have antifreeze activity and also it has probable function of the protein may be to bind a ligand other than ice, function as apolipoprotein, coincidentally (Gauthier et al., 2008).

Apolipoproteins are components of plasma lipoproteins that transport various lipids in the blood and other tissue fluids. On amino acid level, mammalian exchangeable apolipoproteins, a multigene family such as ApoAs, ApoCs and ApoE, are well characterized by common structural features of multiple internal repeat units of 22 amino acids (tandem array of two 11 mer) which has been suggested to be a structure element that builds an amphipathic alpha-helix, and also their gene organizations such as intron/exon numbers and locations are well conserved (Li et al., 1988; Segrest et al., 1992). In fish, several apolipoproteins have been identified as counterparts of mammalian apolipoproteins (Delcuve et al., 1992; Babin et al., 1997; Llewellyn et al., 1998; Poupard et al., 2000; Kondo et al., 2001; Concha et al., 2003; Johnston et al., 2008; Chen et al., 2009; Choudhury et al., 2009). Although amino acid sequences of fish apolipoproteins show low similarity with their mammalian counterparts, the common structural features of multiple internal repeat units are also conserved in fish. In contrast, the gene organizations of zebrafish ApoE and pufferfish apolipoprotein multigene family are well conserved and similar to those of mammalian apolipoproteins (Durliat et al., 2000; Kondo et al., 2005). Although the genomic structures of fish AFPIVs have not been reported until now, we assumed that fish AFPIVs might also have conserved genomic structures like apolipoproteins if the probable function of fish AFPIV is an apolipoprotein or at least they are evolved from apolipoproteins as proposed.

During the transcriptome analysis of the Antarctic silverfish, *Pleuragramma antarcticum* (Pa), we identified a putative homolog gene of fish AFPIV. Furthermore, we also obtained a homolog gene from Antarctic black rock cod, *Notothenia coriiceps* (Nc). In this study,

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we cloned and sequenced AFPIV cDNAs from two Antarctic notothenioid fishes whose products are highly similar to MoAFPIV, and also determined their genomic structure. Nowadays, a large number of fish ESTs have been reported (Douglas et al., 1999; Clark et al., 2003; Linney et al., 2004; Rise et al., 2004; Sarropoulou et al., 2005; Douglas et al., 2007; Williams et al., 2007; Brown et al., 2008; Chen et al., 2008; Park et al., 2009; Leong et al., 2010), so two AFPIVs sequences and gene organizations were compared to diverse fishes to elucidate the biological function and evolution of fish AFPIV genes. In addition, we examined antifreeze activity, including thermal hysteresis (TH) activity and ice crystal morphology, using recombinant AFPIVs of the two Antarctic fishes.

2. Materials and methods

2.1. Molecular cloning of cDNA and sequencing of genomic DNA

Total RNA and genomic DNA were isolated from liver tissues of Pa and Nc, using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Purified total RNA of Pa was sequenced using the pyrosequencing methods of DNA Link, Inc. (Seoul, Korea). Among the draft version of assembled Pa contigs, we identified a contig sequence showing high similarity to MoAFPIV, with a short 5′-UTR, an ORF, and a partial 3′-UTR. To confirm the exact sequence, we designed a specific primer set (forward, PaAFP4F; reverse, PaAFP4R; Table 1) based on this contig sequence and used it for polymerase chain reaction (PCR) amplification of the ORF from first-strand cDNA that had been synthesized from 2 μg of Pa liver total RNA by using oligo (dT) primer and Superscript III (Invitrogen). The PCR product was cloned into TOPO TA cloning vector (Invitrogen) and sequenced. Similarly, we performed PCR using the same primer set and cDNA reverse-transcribed from Nc liver total RNA, and the PCR product was cloned and sequenced. The PaAFPIV and NcAFPIV cDNAs were completed by 5′- and 3′-rapid amplification of cDNA ends (RACE), using several primers based on the Pa and Nc ORF cDNA sequences, respectively (Table 1). RACE-PCR was performed using a CapFishing full-length cDNA Premix kit (Seegene, Seoul, Korea), according to the manufacturer's protocol. The complete cDNA sequences of PaAFPIV and NcAFPIV were deposited in GenBank (Accession nos. HM800727 and HM800728, respectively). Genomic DNA fragments containing both AFPIV genes were obtained via PCR using each primer set (Table 1), based on Pa and Nc 5′-UTR and 3′-UTR cDNA sequences, and each isolated genomic DNA as a template. Each amplified DNA fragment was cloned and sequenced. The genomic sequences were deposited in GenBank (HM800729 for Pa and HM800730 for Nc).

Table 1
Primers used in this study.

Name	Sequence (5′→3′)
Target PCR	
PaAFP4F	ATGAAATTCTCCCTCATCG
PaAFP4R	TTAGTTGTCGATGGGTGCG
RACE-PCR	
Pleuragramma antarcticum	
Pa5Race	CGTTCACGAAGGTCGTAGCCTGGTTG
Pa3Race	CAAGATGGTTCTGGACCTGACCGA
Notothenia coriiceps	
Nc5Race	CTCATTCACGAAGCTCGTAGCCTGG
Nc3Race	CAAGATGGTTCGGGAGCTGACCGA
Expression	
eAFPF	GGATCCATGAAATTCTCCCTCATCG
ePaAFPR	CTCGAGTTAGTTGTCGATGGGTGCG
eNcAFPR	CTCGAGTTAGTTGGCGATGGGTGCG
Genomic DNA PCR	
PaAFPgF	ACCATCTGGTCCAGTTTTCTTC
NcAFPgF	ACCATCCGGTCCAGTTTTCTCC
AFPgR	TGGTGTGGCACATCTGCAT

2.2. Sequence and phylogenetic analyses

The signal sequence and putative cleavage site were identified using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP). AFPIV-like sequences from diverse fishes were obtained by blastX using the PaAFPIV nucleotides as a query of GenBank non-redundant proteins and tblastX on EST databases (Table 2). We reexamined the ESTs obtained via computational translation and BlastP analysis in the case of an existing number of similar ESTs in one fish species and we selected one with a relatively full-length transcript. The genome sequences containing AFPIV-like genes were obtained from finished or/ongoing Ensembl fish genome databases. Multi-sequence alignments were performed using the CLUSTAL W algorithm and a phylogenetic tree was built using the neighbor-joining method via 1000 replicates with MEGA (ver. 4; Tamura et al., 2007).

2.3. Expression, purification, and characterization of recombinant AFPIVs

The coding regions of PaAFPIV and NcAFPIV were amplified by PCR, using forward primers that included a *Bam*HI site (eAFPF for Pa and Nc) and reverse primers that included an *Xho*I site (ePaAFPR for Pa and eNcAFPR for Nc; Table 1). The PCR products were cloned into TA cloning vector and sequenced. The corresponding plasmids were isolated, digested with *Bam*HI and *Xho*I, purified from agarose gels, and ligated into the expression vector pET28a(+) (Novagen), which had been digested with *Bam*HI and *Xho*I. The ligation mixtures were transformed into DH5α competent cells. The purified plasmids, designated pPaAFPIV

Table 2
Accession numbers of AFPIV or AFPIV-like sequences from the GenBank or Ensemble databases.

Species	Abb.	mRNA	Genomic DNA	Notes
Carassius gibelio	CG	AY365004		
Danio rerio	DR	AY345904	ENSDARG00000041490	
Dicentrarchus labrax	DL	FM023094		
Dissostichus mawsoni	DM	FE219242		
Gadus morhua	GM	AY584595		
Gasterosteus aculeatus	GA	EG591368	ENSGACG00000003808	GA1[a]
			ENSGACG00000013154	GA2[a]
Hippoglossus hippoglossus	HH	EB032602		
Lithognathus mormyrus	LM	EB510349		
Monopterus albus	MA	GW584499		
Myoxocephalus octodecemspinosus	MO	AF026525		
Myoxocephalus scorpius	MS	EU542700		
Notothenia coriiceps	NC	HM800728	HM800730	
Oncorhynchus mykiss	OM	CX148905		
Oryzias latipes	OL	DK052473	ENSORLG00000016252	
Osmerus mordax	RS	CX350230		
Paralichthys olivaceus	PO	[b]	[b]	
Pimephales promelas	PP	DT360238		
Platichthys flesus	PF	EC378425		
Pleuragramma antarcticum	PA	HM800727	HM800729	
Psetta maxima	PM	EY455070		
Pseudopleuronectes americanus	WF	AW013035		
Salmo salar	SS	BT046800		SS1[c]
		BT057620		SS2[c]
Sparus aurata	SA	CV133476		
Takifugu rubripes	TR	AL837299	ENSTRUG00000004297	
Tetraodon nigroviridis	TN		ENSTNIG00000009154	

Abbreviations are used for genomic sequences in Fig. 3, and for amino acids in Figs. 4 and 6.
[a] GA1 and GA2 (numbering is arbitrary) are separated genomes of *G. aculeatus*.
[b] Unpublished data.
[c] SS1 and SS2 (numbering is arbitrary) are distinct transcripts in *S. salar*.

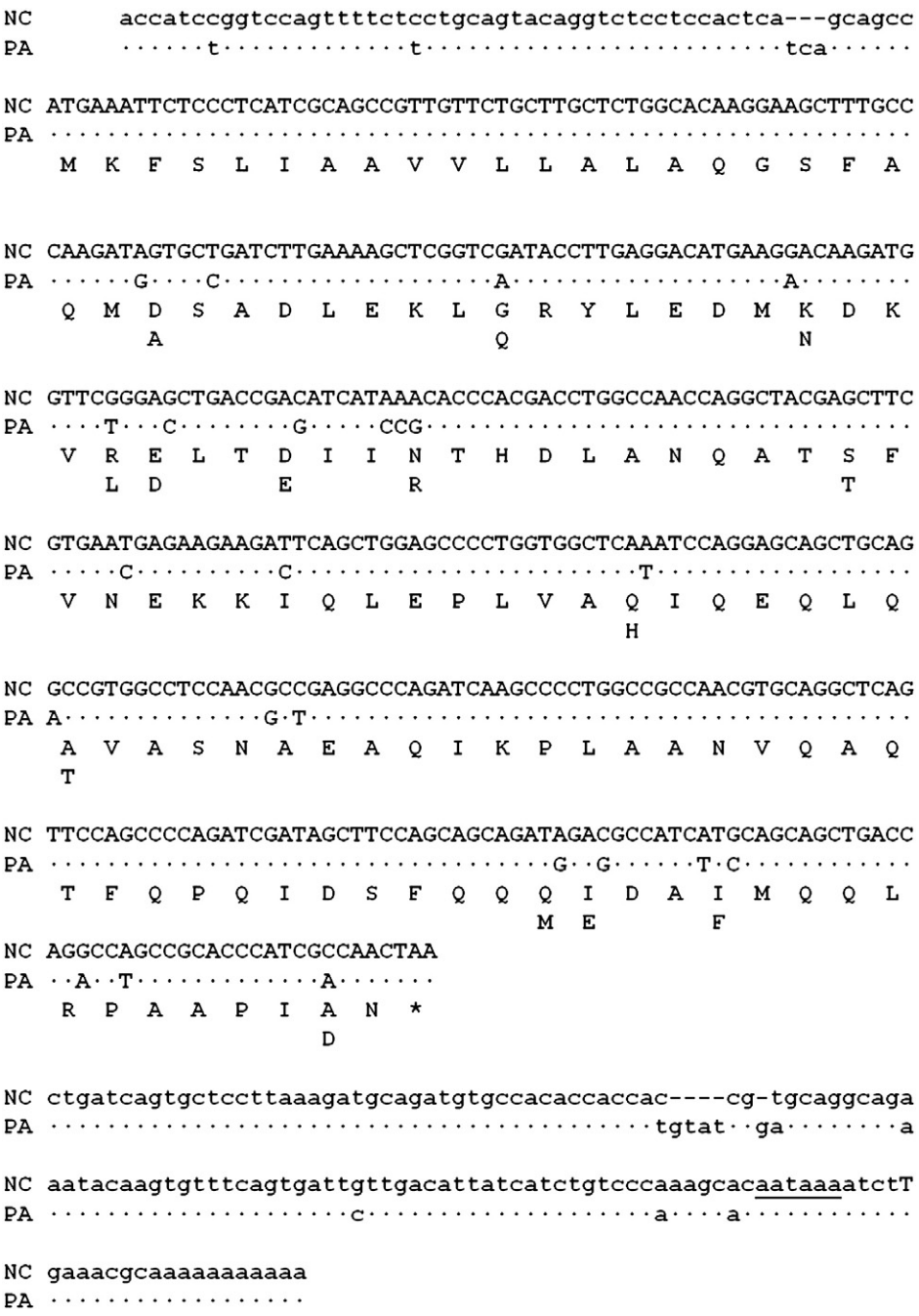


Fig. 1. Nucleotide sequences of NcAFPIV and PaAFPIV. Identical nucleotides of PaAFPIV with NcAFPIV are indicated as dots. Dashes are inserted for alignment. The deduced amino acids of NcAFPIV and PaAFPIV are represented under the nucleotide sequences, where the different amino acids of PaAFPIV to NcAFPIV are represented. The stop codon is marked with an asterisk. Lowercase letters are non-coding regions. The putative polyadenylation signal (AATAAA) is underlined.

and pNcAFPIV, were transformed into BL21 (DE3) competent cells. For each recombinant plasmid, a single positive colony was inoculated into 5 mL of Luria-Bertani (LB) medium supplemented with kanamycin (30 μg/mL) and cultured at 37 °C overnight with shaking. Then, 1 mL of the culture was transferred into 1 L of LB broth containing kanamycin (30 μg/mL) and cultured at 37 °C. When the optical density at 600 nm (OD_{600}) was 0.5, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 3 mM, to induce expression. The culture was incubated for another 6 h at 37 °C with shaking. The cells were harvested by centrifugation (5000 *g*, 30 min, 4 °C), suspended in lysis buffer (20 mM Tris–HCl, pH 7.4, 0.5 M NaCl, 1% Triton X-100, 50 mM PMSF), and sonicated for 2 min on ice (5-s pulse, 10-s delay). After centrifugation (16,000 *g*, 10 min, 4 °C), the upper soluble fraction was transferred to a new tube. The insoluble pellet was resuspended with lysis buffer. The soluble and insoluble fractions were analyzed by SDS-PAGE. The recombinant AFPIVs were purified from 4.5 mL of the soluble supernatant by Ni-affinity chromatography on a HiTrap chelating HP column (GE Healthcare, Waukesha, WI, USA) equilibrated with binding buffer (20 mM sodium phosphate, pH 7.4, 0.1 M NaCl, 5 mM imidazole). After several washings with binding buffer, the protein was eluted, and the eluent was concentrated by ultrafiltration through a YM1 filter (molecular mass cutoff, 1000 Da) in a stirred cell (8050; Millipore, Billerica, MA, USA).

Thermal hysteresis (TH) activity and ice crystal morphology were examined using a nanoliter osmometer (Otago osmometers) as described previously (Lee et al., 2010).

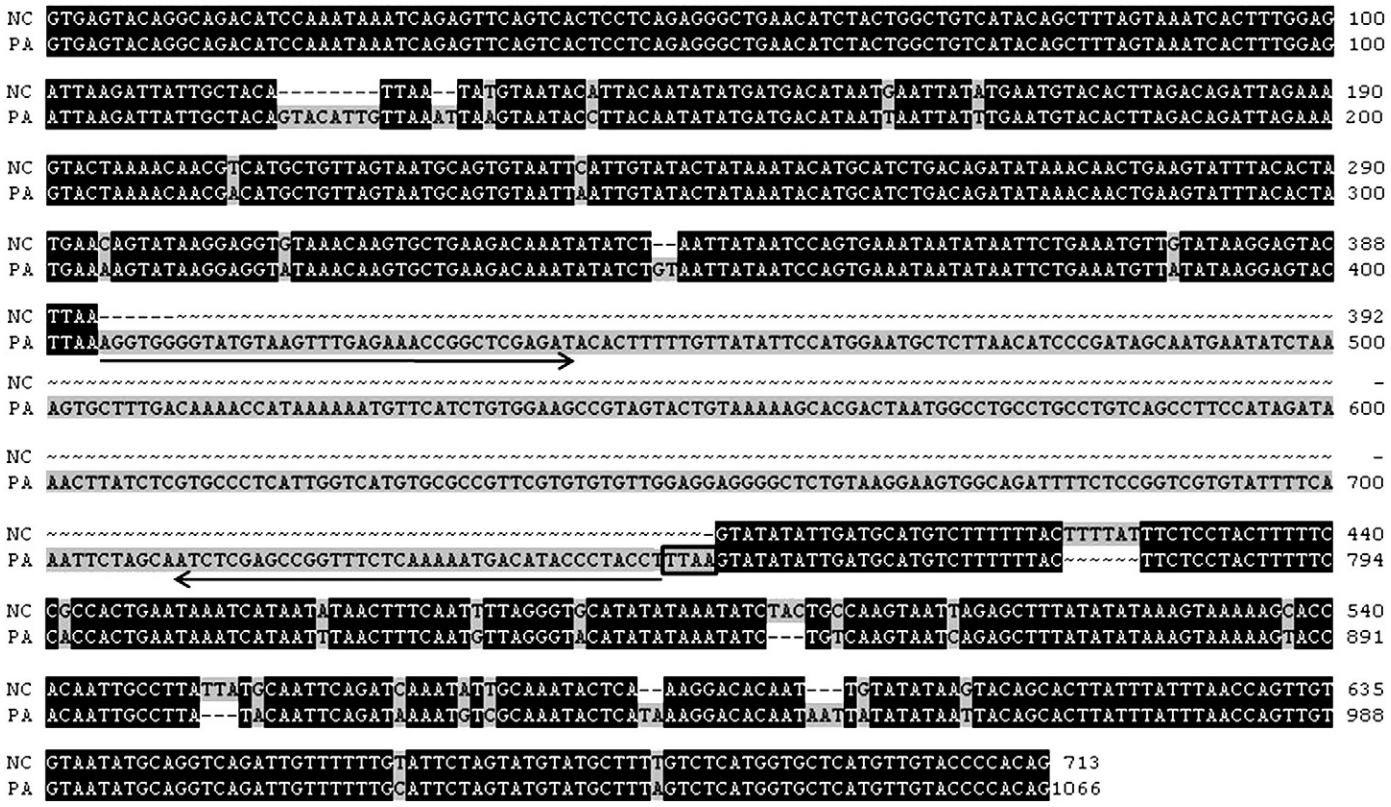


Fig. 2. Comparison of intron 3 sequences of PaAFPIV and NcAFPIV. Arrows indicate the terminal inverted repeat (TIR). Duplicated nucleotides (TTAA) are boxed.

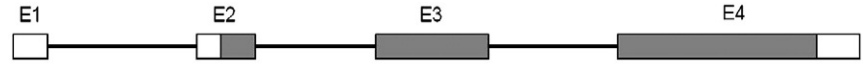
3. Results

3.1. Cloning and sequence analysis of PaAFPIV and NcAFPIV cDNAs

The nucleotide sequence of PaAFPIV was composed of 55 nt in the 5′-UTR, 128 nt in the 3′-UTR, and a 387 nt ORF encoding a protein of 128 amino acid residues. The nucleotide sequence of NcAFPIV was composed of 52 nt in the 5′-UTR, 122 nt in the 3′-UTR, and a 387-nt ORF encoding 128 amino acids (Fig. 1), with 94% similarity between the NcAFPIV and PaAFPIV, and also 83% similarity between each two AFPIVs and AFPIV of the longhorn sculpin, *M. octodecemspinosus*, respectively. The NcAFPIV nucleotide sequence showed 93% identity with that of PaAFPIV. In both fishes, the polyadenylation signal (AATAAA) was conserved between 14 and 18 nt upstream of the polyadenylation site. The molecular masses of PaAFPIV and NcAFPIV precursor proteins were calculated to be 14,205 and 14,118 Da, respectively. The SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP) predicted a secretion signal peptide of 20 amino acid residues in PaAFPIV and NcAFPIV (Bendtsen et al., 2004).

3.2. Genomic structure of PaAFPIV and NcAFPIV genes

To obtain genome sequences, the PaAFPIV and NcAFPIV genomic DNA fragments were amplified by PCR using specific primers and isolated genomic DNAs. The exon/intron structure was analyzed via alignment of genomic sequences with corresponding cDNA sequences. Four exons and three introns were highly conserved in the two fishes. The second exon encoded peptides containing a few residues of the signal peptide, unrelated coding region 1 (UCR1), and a 33-codon block (repeats 1 to 3). The third and last exon encoded the remaining mature proteins, which consisted of seven 11-mer repeat units (4–9) and UCR2. The intron positions were perfectly conserved, with the first large intron located in the 5′-UTR. Furthermore, the intron sequences were highly similar: intron 1 showed 92% identity,



PA	32	1343	23	49	97	124	1066	214	39
NC	32	1325	20	49	97	124	713	214	39
PO	31	1389	20	49	117	124	701	202	175
GA1	*	*	*	49	111	124	229	214	134
TN	31	627	20	49	89	91	76	196	99
TR	31	662	17	49	90	91	85	202	163
DR	31	130	24	46	2004	127	106	220	101
OL	33	95	23	52	294	151	96	262	77
GA2	*	*	*	52	85	151	577	262	*

Fig. 3. Schematic representation of genomic structures from the AFPIV genes of teleost fishes. Refer to Table 2 for GenBank Accession numbers and abbreviations. Exons and introns are indicated by the boxes and lines, respectively. The boxes in gray show the coding region, the empty boxes show the untranslated region. The nucleotide sizes of each exon and intron are represented along with the species. *indicates genome sequences for which the exact sizes could not be calculated due to the absence of transcript sequences.

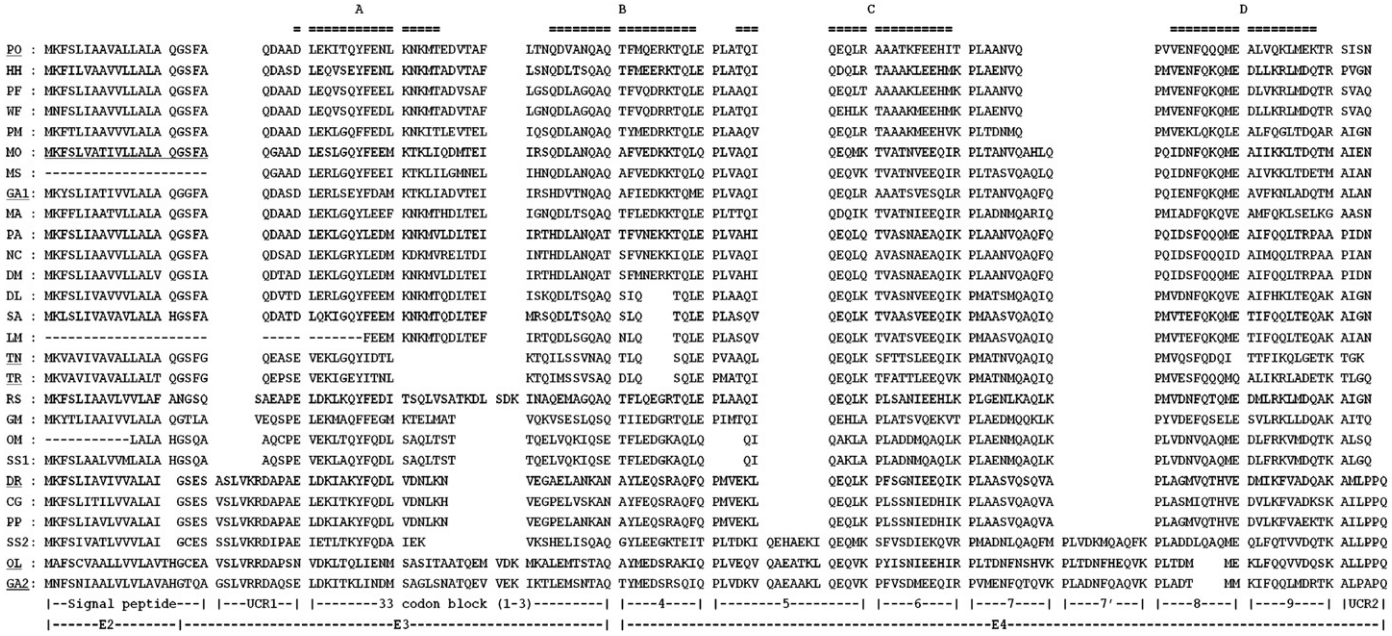


Fig. 4. Multi-alignment of fish AFPIVs. Refer to Table 2 for GenBank Accession numbers and abbreviations. The underlined abbreviations indicate ones for which the genomic sequence is available. All signal peptides except underlined MO are putative. Spaces are introduced for alignments. The dashes indicate unknown regions. Each exon (E2, E3, and E4) and the boundaries of the signal peptide (signal peptide), unrelated coding region 1 and 2 (UCR1 and UCR2, respectively), the 33-codon block, and 11-mer repeats (4 to 9) are indicated under the alignments.

Fig. 5. Comparison of helix wheels of four helices (A, B, C, and D) of AFPIVs from four fishes. Inner to outer circle: MO, MS, PA, and NC. Arrows at each helix indicate the beginning amino acid and direction of the helix. Yellow, nonpolar; Green, uncharged polar; Blue, + charged; Red, − charged amino acids.

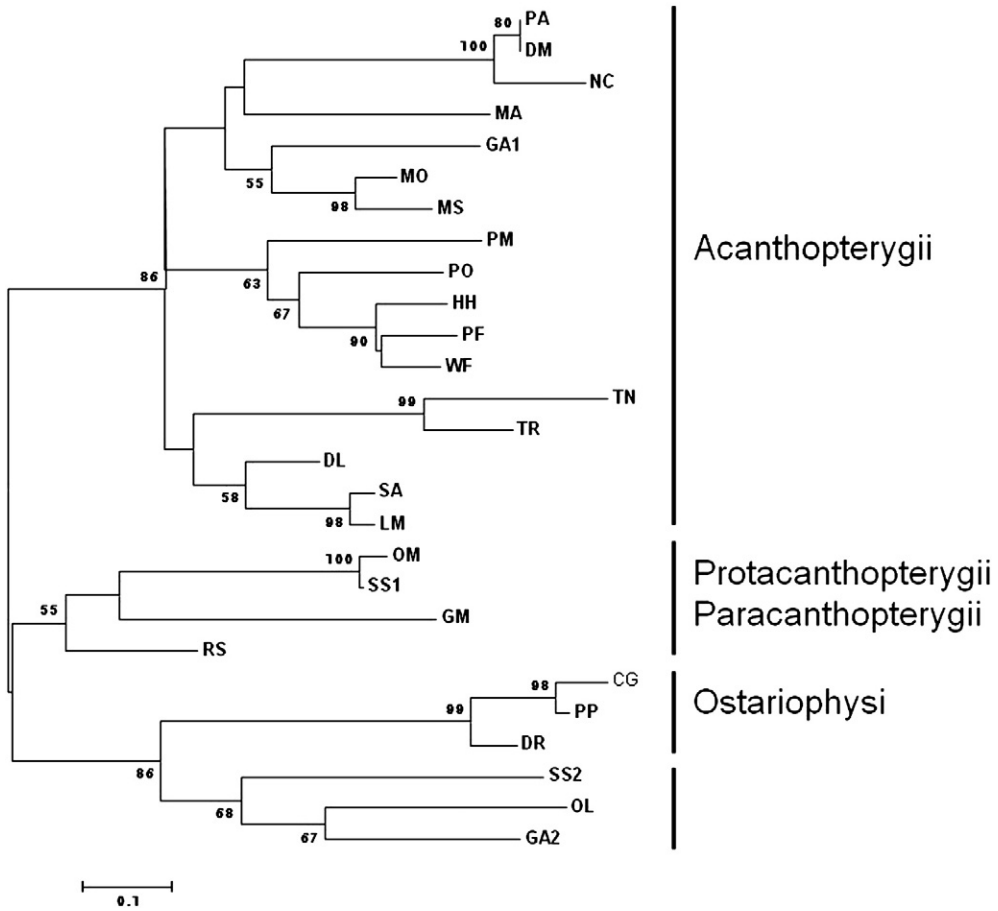


Fig. 6. Phylogenetic tree of fish AFPIVs. See Table 2 for abbreviations. The unrooted tree was built using the neighbor-joining method, bootstrapped with 1000 replicates. Only bootstrap values above 50% are shown on the branches.

intron 2 showed 96% identity, while intron 3 of PaAFPIV contained additional nucleotides with terminal inverted repeats (TIRs) including four direct nucleotide duplications (TTAA) at both ends (Fig. 2). After removing the inserted sequence of PaAFPIV, intron 3 also showed a high identity (95%). The nucleotide sequence alignment revealed that their TIRs contained the T2-like motif, TTAAAGGTG (Unsal and Morgan, 1995). They showed non-perfect palindromic structures (data not shown), similar to the *Angel* element, which is a miniature inverted-repeat transposable element (MITE) found in zebrafish (Izsvak et al., 1999). A comparison of the genome structures of fish AFPIV genes revealed that the intron positions were well conserved, while the intron sequences and sizes varied among the fishes examined (Fig. 3).

3.3. Amino acid sequence analysis of type IV AFPs

Multiple alignments of amino acid sequences were conducted between PaAFPIV and NcAFPIV with other fish AFPIVs, using the CLUSTAL W algorithm. Subsequently, it was manually edited, based on the signal peptides, exon 2-coding regions, and exon 3-coding regions. All signal peptides examined, except for MoAFPIV, were predicted by SignalP. Proteins with no available genome sequence were aligned based on sequence similarity (Fig. 4).

Using this approach, Signal peptides, UCR1, a 33 codon block (1–3), seven 11-mer repeat units (4–9), and UCR2 were well conserved among fish AFPIVs with some minor variations. The most variable region was 33 codon block, especially in repeat 2. Repeat 2 was completely absent in TN and TR, or several amino acids in repeat 2 were deleted or inserted in some fishes. In repeats 4, 7, and 8 of some fishes, 4 amino acids were deleted. In repeat 5, four-residue deletions or eight-residue insertions were detected. The additional repeat unit (7′) was inserted in SS2, OL, and GA2. The first residue, P, of repeat 6 was substituted by other amino acids, mostly A and T. From the last 11-mer repeat of the 33 codon-block to repeat 9, the most conserved amino acid was the 9th residue, Q, in each 11-mer repeat. Deng and Laursen, 1998 proposed that the antiparallel bundle model consisted of four amphipathic alpha-helices (A, B, C, and D). There is a linker (CD) between the C and D helices. According to this model, helix A resides on the first and second 11-mer repeat of the 33-codon block; helix B on the third 11-mer repeat of 33-codon block and repeat 4; helix C on repeat 5 and 6; linker (CD) on repeat 7; helix D on repeat 8 and 9. Thus, each helix contains two 11-residue repeats. Observed variations were generally positioned in the loop regions between helices. Fig. 5 shows helix wheel representations of the four helices (A, B, C, and D) of the four fishes including PaAFPIV and NcAFPIV in which antifreeze activity was actually examined. The hydrophobic core is highly conserved in each helix. However, no interhelical salt bridge was observed between helices A and D, or between helices C and D in PaAFPIV or NcAFPIV, because the K_{60} in helix C and K_{98} in helix D of MoAFPIV and MsAFPIV are substituted with the uncharged amino acid, Q.

3.4. Phylogenetic analyses of PaAFPIV and NcAFPIV

AFPIV-like genes were found in 25 fishes of 14 orders belonging to the class Actinopterygii. Most are temperate fishes, while there are also tropical or subtropical fishes, such as the zebrafish. Despite some sequence variations, all proteins examined in this study were grouped together, separated from other apolipoprotein groups in phylogenetic

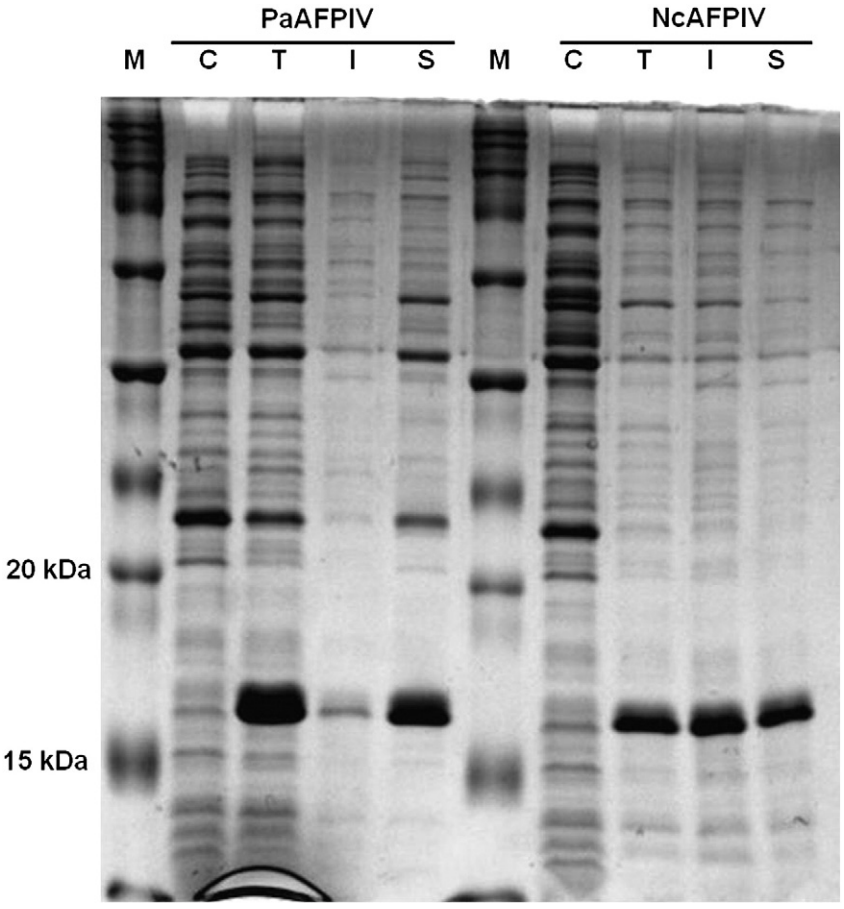


Fig. 7. Expression of recombinant PaAFPIV and NcAFPIV in *E. coli*. Lanes: C, Total cell lysate containing vector only; T, total cell lysate; I, insoluble fraction; S, Soluble fraction; M, size marker.

analysis (data not shown). The phylogenetic tree of fish AFPIV-like genes showed that they were divided into three clades in the higher taxons of fishes (Fig. 6). Clade A was almost all composed of superorder Acanthopterygii. Protacanthopterigii (Salmoniformes and Osmeriformes) and Paracanthopterigii (Osmeriformes) were included in Clade B. Clade C contained mainly Ostariophysi (Cypriniformes), Salmoniformes, and Gasterosteiformes.

3.5. Expression, purification, and characterization

Fig. 7 shows that recombinant PaAFPIV and NcAFPIV were expressed in soluble and insoluble forms. The apparent molecular mass of each recombinant protein was approximately 17 kDa, based on a calculated molecular mass of 17.7 kDa, which includes 34 vector-related residues such as the 6-His tag at the N-terminus. The purified AFPIVs were concentrated by ultrafiltration. However, precipitation and aggregation occurred at concentrations of more than 0.5 mg/mL, which is similar to the behaviors of recombinant MoAFPIV and MsAFPIV (Gauthier et al. 2008). PaAFPIV and NcAFPIV gave ice crystals with star-shaped morphology (Fig. 8), and their TH values were 0.08 °C at the concentration of 0.5 mg/ml. The N-terminal vector-related residues of the recombinants might have reduced their solubility, although we did not examine the solubility of native PaAFPIV and NcAFPIV.

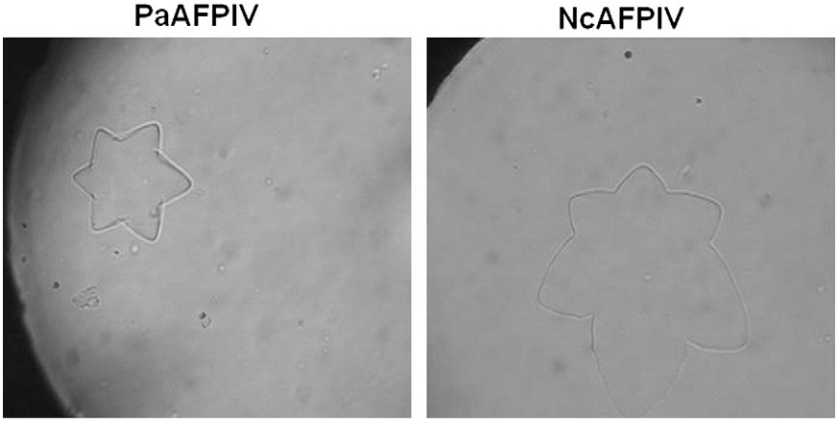


Fig. 8. Ice crystal growth morphology of PaAFPIV and NcAFPIV (each at 0.5 mg/mL).

4. Discussion

Since the incidental discovery of the antifreeze activity of fish type IV AFP from the longhorn sculpin (Deng et al., 1997), the main function of this protein is still obscured by antifreeze activity and/or apolipoprotein which is due to the common primary structures (Cheng 1998). In this paper, we cloned and expressed AFPIVs from two Antarctic notothenioid fishes for the first time. The recombinant form of PaAFPIV and NcAFPIV showed antifreeze activity and similar properties to recombinant MoAFPIV and MsAFPIV (Gauthier et al. 2008). Comparative analyses of identified AFPIV homolog genes from many fishes were performed at nucleotide sequences and genomic structure. It was observed that fish AFPIVs are widely distributed genes in teleostei from tropic, subtropic, temperate and polar fishes, and also these sequences were well accordance with both common structural features of multiple internal repeat units and gene organizations of fish apolipoproteins. The molecular masses of fish AFPIVs were the most similar to that of fish 14 kDa apolipoprotein among the other fish and mammalian apolipoproteins. 14 kDa Apolipoproteins were identified in diverse fishes and it is thought to be fish-specific apolipoprotein (Kondo et al., 2001; Kondo et al., 2005; Zhou et al., 2005; Xia et al., 2008; Kim et al., 2009). However, the amino acid alignment between fish AFPIVs and 14 kDa apolipoproteins and phylogenetic analysis showed that they are distinct each other. Also there are some reports that the AFPIV and 14 kDa apolipoproteins transcripts were shown in different expression patterns according to developmental stages. Liu et al., 2009 reported that the AFPIV gene in gibel carp (*Carassius auratus gibelio*) was highly expressed during early life stages, and declined after larval stages, while 14 kDa apolipoprotein of orange-spotted grouper (*Epinephelus coioides*) were expressed stably during embryogenesis and also continued predominantly in adult life stage (Zhou et al., 2005). Therefore, AFPIVs do not appear to be major proteins in the lipid transport system, because of their low concentrations in the blood of adults (Deng et al. 1997; Gauthier et al. 2008). It is uncertain whether all examined AFPIV-like proteins with deleted or inserted variations relative to MoAFPIV have coincidental antifreeze activity. However, whatever their biological roles, AFPIVs having coincidental antifreeze activity could provide an advantage by protecting the embryo from freezing in icy waters before the circulatory blood system is generated during later life stages. It could also be assumed that this would be helpful for fishes to evolve and adapt to cold environments before obtaining active antifreeze proteins, such as other types of fish AFPs.

In conclusion, we report here the existence of AFPIV genes in two Antarctic fishes and antifreeze activity of their recombinant proteins when produced in *E. coli*. Although PaAFPIV and NcAFPIV do have antifreeze activity, the antifreeze activities were very low compared to other type AFPs. Future studies should address its definite roles in these fishes, we regard that the coincidental antifreeze activity of fish AFPIVs may be helpful in adapting to cold environments.

Acknowledgements

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