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博士号論文

Studies on molecular mechanisms underlying high pressure adaptation of α-actin from deep-sea fish

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Abstract Deep-sea fish distribute to depths of several thousand meters and at these abyssal depths encounter pressures that shallower-living fish cannot tolerate. Tolerance to abyssal pressures by deep-sea fish is likely to depend at least in part on adaptive modifications of proteins. However, structural modifications that allow proteins to function at high pressures have not been well elucidated. The objective of this study is to disclose the mechanisms underlying adaptation of deep-sea fish to high pressures. First, in order to select sample fish for this study, the author constructed the molecular phylogenetic trees for the deep-sea fish *Coryphaenoides* using the nucleotide sequences of the mitochondrial 12S rRNA and COI genes. The trees showed new arrangements of seven *Coryphaenoides* species with distinct groups, abyssal and non-abyssal species, that differed from previous taxonomic studies. Using the mutation rate of mitochondrial genes, the divergence time between abyssal and non-abyssal *Coryphaenoides* was calculated to be 3.2-7.6 million years ago. The present study suggests that hydraulic pressures play an important role in the speciation process in the marine environment. Second, the author cloned cDNAs encoding α-actin, which was used as a model protein to elucidate the mechanisms involved in protein adaptation to high pressures, from two abyssal *Coryphaenoides* species, *C. armatus* and *C. yaquinae*. Consequently, the author identified three amino acid substitutions, V54A or L67P, Q137K and A155S, that distinguished these abyssal α-actins from orthologs from non-abyssal *Coryphaenoides*. Finally, the author examined by several biochemical analyses which of the three substitutions makes possible for α-actin of the deep-sea fish adapt to high hydrostatic pressures. It was found that the substitutions of Q137K and A155S prevent the dissociation reaction of ATP and Ca^{2+} from being influenced by high pressures. In particular, the substitution of Q137K results in a much smaller change in the apparent volume for Ca^{2+} dissociation reaction. The substitution of V54A or L67P reduced the volume change associated with actin polymerization and has a role in maintaining the DNaseI activity of actin at high pressures. Taken together, these results indicate that a few amino acid substitutions in key functional positions can adaptively alter the pressure sensitivity of abyssal proteins.

Key Words: deep-sea fish, high pressure adaptation, actin, *Coryphaenoide*, molecular phylogenetic tree

Contents

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Introduction

The oceans constitute the largest habitat on Earth. Seawater covers 71% of its surface to an average depth of 3800m. Continental shelves in the region of 0 to 200m depth cover approximately 5 % of the Earth's surface, whereas slopes of 200 to 3000m depth, abyssal areas of 3000 of 6000m depth, and hadal areas of > 6000m depth do 13 %, 51 % and < 2 %, respectively (Fig. 1). The total volume of the oceans is $1.368 \times 10^9 km^3$, providing living space estimated to be 168 times that offered by terrestrial habitats (Cohen, 1994). The immense volume of seawater contributes 0.24% of the total mass of Earth and has a major influence on its climate.

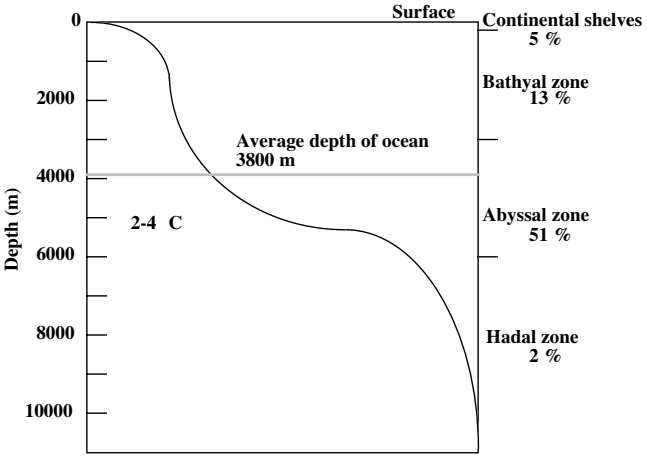

Fig. 1. Classification of the marine environments. The percentages refer to all Earth's surface area of the ocean floor included in each depth zone.

The deep-sea, defined variously as the ocean depths of 200 to 1000m, had been believed to be stagnant without life for a long time. But studies in the later 18th century revealed that it contains an abundance of life, often of bizarre forms (Mills, 1983). Though recent studies have clearly indicated that much more dynamic habitat exists than commonly believed (Gage and Tyler, 1991), the deep-sea had been considered to be a mysterious realm and organisms had been rarely explored.

The deep-sea is characterized by low temperature (1-4℃), extremely high hydrostatic pressures, non-photosynthetic light, and the relatively low influx of utilizable organic materials derived from the primary production in surface waters. These environmental factors endow organisms with some problems in colonizing this habitat. While some fish inhabit under the particular hydrostatic pressure, another experience various hydrostatic pressures during their life history. For example, certain fish spend early development stages in more productive area such as shallow waters, whereas vertical migration is prerequisite for reproduction of other fish species (Merret, 1978). However, most organisms inhabiting the deep-sea apparently have a characteristic bathymetric distribution throughout its geographic range. Therefore, the hydraulic pressure is considered to be especially important in the deep-sea life.

The hydrostatic pressure increases by 0.1 mega pascal (MPa) with the increase of every 10m depth in the ocean (Saunders and Fofonoff, 1976). Several different measurement units for pressure have been used in the literature. Recently the System International (SI) unit, Pa, has been authorized as a unit for pressure. The following conversion factors can be used: 1 atm = 1 bar = 14.7psi = 101325Pa. Hydrostatic pressures influence the function of proteins and biological membranes, especially in complex processes such as protein-protein interactions, enzyme-substrate binding, lipid-protein interactions, signal transduction, and biochemical correlates of organismal metabolism (Somero, 1990, 1992). Thus, hydrostatic pressures are considered

to play the critical role in vertical distribution of organisms and speciation in the deep sea, where homologous proteins from deep-sea fish tend to exhibit insensitivity to hydrostatic pressures (Siebenaller, 1991; Siebenaller and Murray, 1995; Childress and Thuesen, 1995; Gibbs, 1995).

Deep-sea fish distribute to abyssal depths of several thousand meters, the pressures of which shallower-living fish cannot tolerate. Tolerance to abyssal pressures by deep-sea fish is likely to depend at least in part on adaptive modifications of proteins. However, structural modifications that allow proteins to function at high pressures have not been well explored.

In the present study, the author made an attempt to give an answer to the classic, not old, and simple question: how do deep-sea fish adapt to high pressures of approximately 60 MPa ?

In Chapter 1, the author constructed the molecular phylogenetic trees of *Coryphaenoides* species by sequence analysis of two mitochondrial (mt) genes, *i.e.*, the 12S rRNA and cytochrome oxidase subunit I (COI) genes. Marine fish *Coryphaenoides* have a widespread bathymetric vertical distribution (Marshall, 1979) and have been extensively studied as model fish to elucidate the mechanisms involved in adaptation to the deep-sea (Smith, 1978; Siebenaller *et al*., 1982; Stein and Pearcy, 1982). An excellent study system for efficiently investigating the environmental adaptation seems to compare the closely related'species inhabiting different environments. A reliable molecular phylogenetic tree will create a way for selecting such sample species. The resent molecular phylogenetic trees based on mtDNA analysis indicated that abyssal *Coryphaenoides*, such as *C. armatus* and *C. yaquinae*, diverged 3.2-7.6 million years ago from non-abyssal species as the first event of *Coryphaenoides* speciation.

In Chapter 2, the author focused on the elucidation of the mechanism underlying high hydrostatic pressure adaptation of deep-sea fish. Many previous studies have identified proteins from deep-sea fish that function at high pressures (Swezey and Somero, 1985; Somero, 1990, 1992; Siebenaller, 1991; Siebenaller and Murray, 1995; Gibbs, 1997; Yancey *et al*., 2001). One of these studies determined the volume change (δV) that is associated with polymerization of globular (G)-to filamentous (F)-state of α-actin from abyssal *C. armatus* and non-abyssal *C. acrolepis* and estimated the δV of actin from *C. armatus* was much smaller to be advantageous for a deep-sea habitat than those from *C. acrolepis* (Swezey and Somero, 1985). Actin is the major component of the microfilament system in all eukaryotic cells and plays a central role in maintaining cytoskeletal structure, cell motility, cell division, intracellular movements and contractile processes (Sheterline *et al*., 1999; Pollard *et al*., 1986). Thus, actin is one of the most conserved proteins in eukaryotic cells (Sheterline *et al*., 1999). For example, the α-actin molecules from carp and rat share 99.4% homology at the amino acid sequence level (Collins and Elzinga, 1975; Watabe *et al*., 1995). It is surprising that differences in the δV of this highly conserved protein have been found between two species of *Corypanenoides* that inhabit different niches. Therefore, the author expected that key amino acid substitution could allow the deep-sea fish to adapt to high hydrostatic pressures, and that such substitution would be easily found in comparison of actins from closely related species of *Corypanenoides* inhabiting different depths with a short divergence time. First, the author purified α-actin as a model protein for investigating high hydrostatic pressure adaptation from fast skeletal muscles of *C. yaquinae*, *C. armatus*, *C. acrolepis*, carp and chicken. Second, he cloned the α-actin cDNAs from *C. yaquinae*, *C. armatus*, *C. acrolepis* and *C. cinereus*, and determined their primary structures. He consequently identified three amino acid substitutions between non-abyssal and abyssal *Coryphaenoides*.

In Chapter 3, based on the structures of the actin molecules determined in Chapter 2 along with those previously reported, the author examined which of the three substitutions enables α-actin of the deep-sea fish to adapt to high hydrostatic pressures by using biochemical analyses, such as Quin 2, nucleotide exchange, and DNaseI inhibition assays. The results obtained indicate that the three amino acid substitutions provided deep-sea fish α

-actin with respective functions to cope with high pressures.

Chapter 4 is devoted to general discussion based on the results of this study and other related ones.

The present findings could contribute to better understanding of the formation of bathymetric distribution for marine organisms and the evolution of deep-sea organisms.

Chapter 1. Molecular phylogenetic relationships of the deep-sea fish *Coryphaenoides* (Gadiformes: Macrouridae)

In order to study efficiently the adaptation of proteins to high hydrostatic pressures, it is useful to compare closely related species inhabiting different depths. The divergence time among the closely related species among orthologs are relatively short. Therefore, it is predicted that a few amino acid variations among orthologs would allow the proteins to adapt to high hydrostatic pressures. An excellent study system for such purposes is provided by marine fish *Coryphaenoides*.

Marine fish *Coryphaenoides* are diverse and have a widespread bathymetric vertical distribution from 200 to 6400m in depth in the ocean. These are abundant in deep sea area of the Pacific ocean, and have been studied as model fish to elucidate their adaptation to the deep sea (Smith, 1978; Siebenaller *et al*., 1982; Stein and Pearcy, 1982). Most *Coryphaenoides* species inhabit a restricted vertical range on continental slopes of 200 to 2000m deep in the ocean, whereas a few species are regarded as abyssal (Marshall, 1979). The present taxonomic arrangement of *Coryphaenoides* defining the subgenera has been based on only a few available taxonomic characters in morphological aspects (Iwamoto and Sazonov, 1988). The largest subgenus is *Coryphaenoides* (46 species), followed by *Chalinura* (8 species), *Nematonurus* (5 species), and *Lionurus* (2 species). The subgenus *Nematonurus* includes abyssal and non-abyssal species. *C. armatus* and *C. yaquinae* in the subgenus *Nematonurus* are known as abyssal species adapting to extremely high hydrostatic pressure among the genus *Coryphaenoides*, and their distribution ranges from 2700 m to 6400m deep in the ocean (Iwamoto and Stein, 1974). Biochemical phylogenetic approaches by peptide mapping of A_4-lactate dehydrogenase (A_4-LDH; 1.1.1.27) isoenzyme to study the evolutional adaptation of *Coryphaenoides* suggest that diversification of this genus began on the upper slope and then spread to the abyssal ocean (Wilson *et al*., 1991; Wilson, 1994) (Fig. 2). Thus, phylogenetic relationships within the *Coryphaenoides* species are still unclear and have been disputed. The selection of candidate organisms considering phylogenetic relationships is important in comparative biochemical and molecular study (Garland and Adolph, 1994; Garland and Carter,

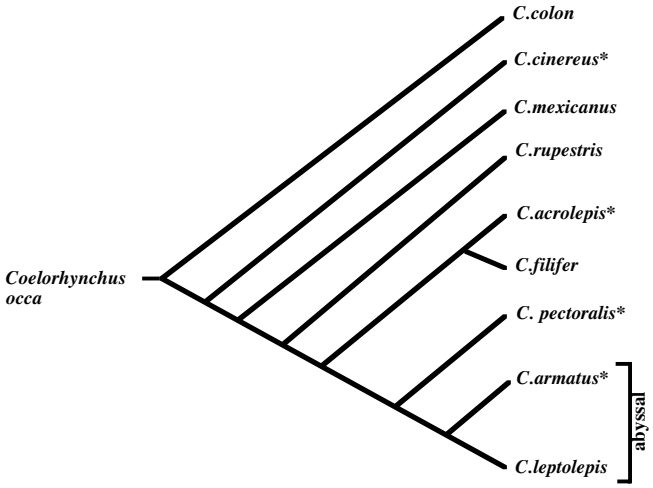

Fig. 2. Phylogenetic tree constructed by least-squares (Fitch-Margoliash) analysis based on peptide mapping of A_4-LDH homologs from *Coryphaenoides* species (Wilson *et al*., 1991). *Species used in this study.

Table 1. Species used in this study

Family / Species	Collection site and depth (m)		
Macrouridae			
(Ingroup taxa)			
Coryphaenoides nasutus	38° 01.76′ N	142° 12.73′ E	810m
C. cinereus	38° 02.13′ N	142° 22.05′ E	997m
C. acrolepis	42° 04.00′ N	144° 42.80′ E	1960m
C. pectoralis	37° 21.35′ N	142° 18.42′ E	1001m
C. longifilis	37° 21.35′ N	142° 18.42′ E	1001m
C. armatus	44° 00.70′ N	145° 22.20′ E	3940m
C. yaquinae	39° 58.10′ N	154° 59.50′ E	5600m
(Outgroup taxa)			
Abyssicola macrochir	37° 58.81′ N	141° 48.97′ E	250m
Caelorinchus gilberti	37° 26.28′ N	142° 00.81′ E	603m

1994). A molecular phylogenetic tree based on the sequence analysis of mtDNA seems more suitable for such purposes (Meyer *et al*, 1990; Normark, 1991; Meyer, 1992; Fabrizio *et al*, 1996; Miya and Nishida, 1996; Ritchie *et al*., 1996; Miya *et al*., 2001).

In this chapter, the author constructed a reliable molecular phylogenetic tree of the genus *Coryphaenoides* by sequence analysis of two mitochondrial genes, *i.e*., the 12S rRNA and COI genes. The molecular phylogenetic tree will provide the guideline for selecting closely related species to investigate the adaptation of proteins from deep-sea fish to high hydrostatic pressures.

Materials and Methods

Materials and DNA extraction

Table 1 summarizes data of species used in this study. *C. armatus* and *C. yaquinae* were collected using large deep-sea pots with long lines by the research vessel (R/V) Soyo-maru of the National Research Institute of Fisheries Science. The other species were collected using trawl nets by the R/V Wakataka-maru of the Tohoku National Fisheries Research Institute. Total DNA was extracted from fast skeletal muscles tissues of frozen specimens by standard protocols using proteinase K digestion and phenol extraction (Kocher *et al*., 1989).

DNA amplification and DNA sequencing

Polymerase chain reaction (PCR) amplifications were performed in 100 μl of 10mM Tris-HCl buffer (pH 8.3) containing 1.5mM $MgCl_2$, 1 mM each of dNTP, 1 mM each of primers, 50-1000ng of template DNA, and 2.5units of Taq polymerase (Takara). The 12S rRNA gene was amplified with a primer set of L1091 and H1478 (Kocher *et al*, 1989). The other set of AAGCTATTATGATGGGCCCT (L640) and GTTCGAGTGAAGTACCATCA (H1110) were newly designed and used in this study. L and H refer to the sequences of light and heavy strands, respectively, and the numbers of the primers indicated the 3' end positions according to the numbering system for human mtDNA (Anderson *et al*., 1981). The COI gene was amplified with primers L6586 and H7086 (Palumbi and Wilson, 1990). PCR was performed at an initial denaturation step at 93 ℃ for 2 min, followed by 30cycles at 93℃ for 40sec, 55℃ for 1min, and 72℃ for 1.5min. The amplified fragments were purified on 1 % low-melting agarose gel. Each purified fragment was determined for its nucleotide sequences by direct sequencing of both sense and antisense strands with the PCR primers used for the DNA amplification as sequence primer. PCR was carried out using a Dye terminator cycle sequencing kit (Applied Biosystems) for 26cycles (at 96℃ for 30sec, 50℃ for 15sec, and 60℃ for 4 min). The cycle sequence products were precipitated with ethanol, resuspended in 4 ml of 50% formamide containing 0.1 M EDTA, and loaded onto an ABI 373A automated DNA sequencer (Applied Biosystems).

All sequences are available from the

DDBJ/EMBL/GenBank databases under accession numbers AB018224-AB018232 for the 12S rRNA gene and AB018233-AB018241 for the COI gene.

Phylogenetic analysis

The nucleotide sequences were aligned with the CLUSTAL W multiple alignment program (Thompson *et al*., 1994) and refined manually. Maximum parsimony (MP) analyses were conducted with the computer program PAUP, test version 4d64 (Swofford, 1998). NEIGHBOR and DNAML in PHYLIP version 3.5 program package (Felsenstein, 1995) was used for neighbor-joining (NJ) (Saitou and Nei, 1987) and for maximum likelihood (ML) (Felsenstein, 1981). Distance matrices for the NJ analysis were estimated by the DNADIST program in the PHYLIP program package with the Kimura's two-parameter option (Kimura, 1980). Transition/transversion (TS/TV) rate was 4.0, according to the observed ratio within the ingroup and between the ingroup and outgroup (Fig. 3). MP was performed by an extensive search of the most parsimonious tree (s). Gaps were treated as missing data in all analyses. Bootstrap analyses (Felsenstein, 1985) were performed to examine the confidence of nodes within the resultant topology obtained by MP, NJ and ML analyses in 1000 times replicates. Abyssicola macrochir and *Caelorinchus gilberti* were used as outgroup species in the MP, NJ and ML analyses.

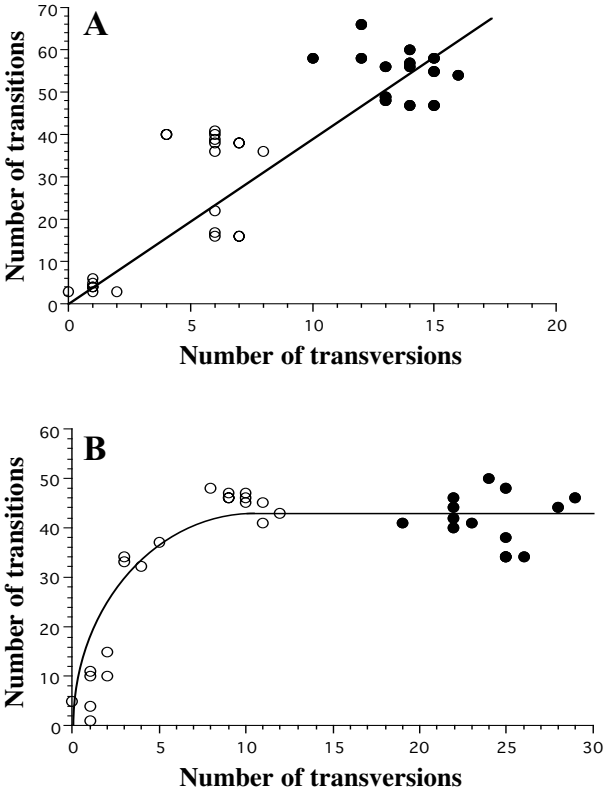

Fig. 3. Levels of saturation for base substitutions in the 12S rRNA and COI genes among the ingroup *Coryphaenoides* species and between *Coryphaenoides* species and outgroup species. (A) 12S rRNA and (B) COI (third codon position) genes. Open symbols indicate pairwise comparisons of transition vs. transversion within the ingroup, and closed symbols indicate those between ingroup and outgroup species.

Results

The nucleotide sequence of the 12S rRNA and COI genes corresponding to positions of 68 to 892 and of 6167 to 6611 of the Atlantic cod gene, respectively (Steinar and Ingrid, 1996) were determined for seven *Coryphaenoides* species (Fig. 4, 5).

The alignment among the determined sequences of the *Coryphaenoides* 12S rRNA gene was 829 bp in length, including 434 bp (52%) of stem sites and 395 bp (48%) of loop sites identified by comparisons with the secondary structure of the bovine 12S rRNA gene (Gutell *et al*., 1985). The base composition among seven *Coryphaenoides* species were A = 22.3%, T = 22.4%, C = 26.7%, and G = 28.7% in the stem site and A = 39.9%, T = 22.4%, C = 22.7%, and G = 14.9% in the loop site. The stems of the fish genes showed a rough base frequencies, while loops had an excess of A and a low frequency of G, as noted in mammals (Springer *et al*., 1995). Several insertions were observed in the loops, but only one insertion in the stems. Pairwise sequence divergence estimates ranged from 0.0036 to 0.0571 (mean = 0.0331) between each two pairs of species within the genus *Coryphaenoides* and from 0.0739 to 0.0947 (mean = 0.0885) in comparison between *Coryphaenoides* and outgroup species (Table 2). TS/TV ratio of the 12S rRNA gene among *Coryphaenoides* species and outgroup species ranged from 1.5 to 10.0 except for *C. pectoralis* and *C. acrolepis* having TV = 0. Since the TS/TV ratio of the 12S rRNA gene within the ingroup was constant,

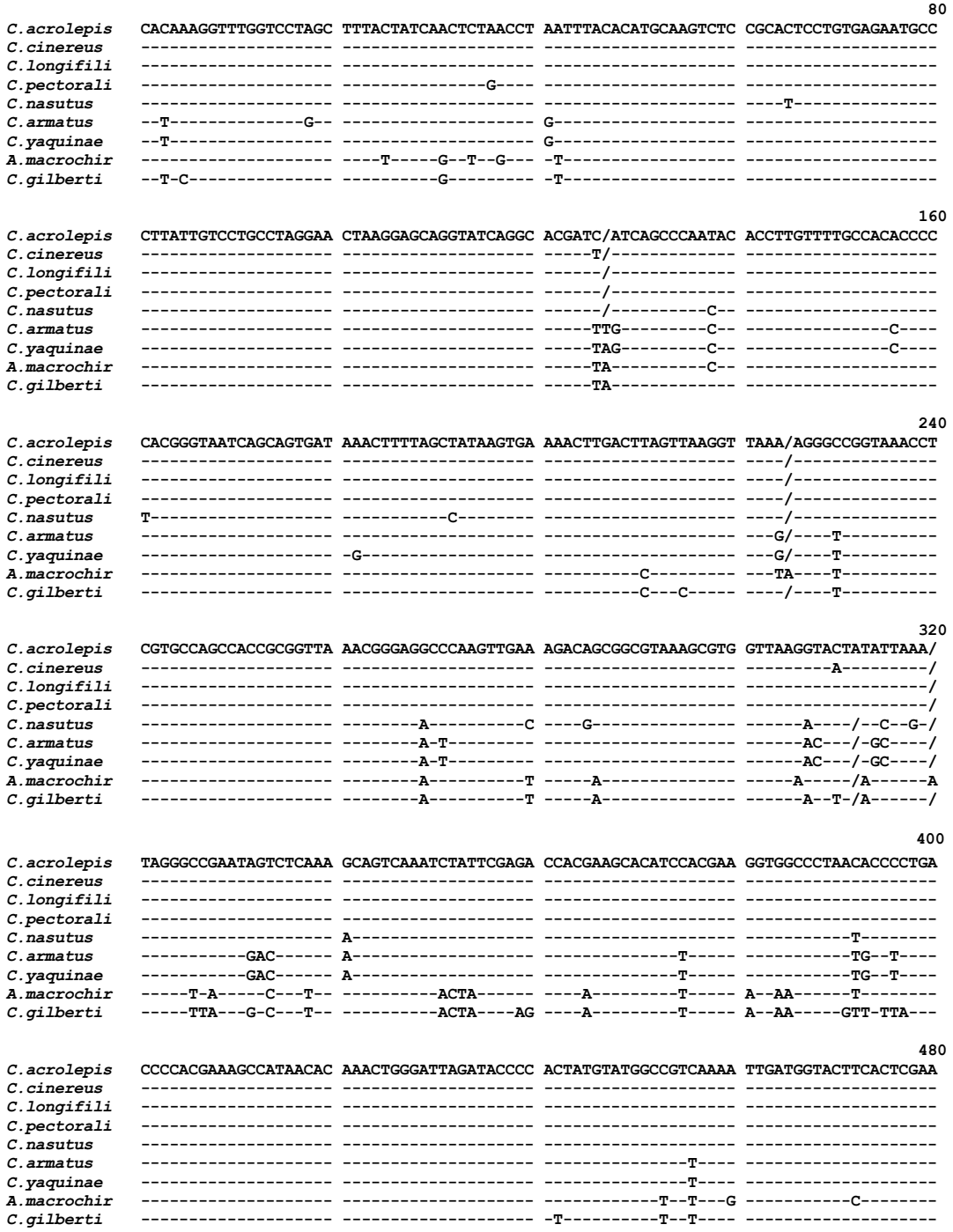

Fig. 4. Comparison of partial nucleotide sequences of the 12S rRNA gene of Coryphaenoides species. A dash indicates an identical nucleotide with that of *C. acrolepis*. The sequences correspond to positions 68-892 of the Atlantic cod homolog (Steinar and Ingrid, 1996). The sequences of Coryphaenoides species are available from the DDBJ/EMBL/GenBank nucleotide databases with accession numbers AB018224-AB018232.

```
                                                                                                                560
C.acrolepis   CCATCCGCCCGGGGACTACG AGCCCTAGCTTAAAACCCAA AGGACCTGGCGGTGCTTTAG ATCCCCCTAGAGGAGCCTGT
C.cinereus    -------------------- -------------------- -------------------- --------------------
C.longifili   -------------------- -------------------- -------------------- --------------------
C.pectorali   -------------------- -------------------- -------------------- --------------------
C.nasutus     ----T--------------A -------------------- -------------------- --------------------
C.armatus     ---------A---A-T---- G----C-------------- -------------------- --------------------
C.yaquinae    ----T----A---A-T---- G----C-------------- -------------------- --------------------
A.macrochir   -------------A------ -----C-------------- -------------------- --------------------
C.gilberti    -------------A------ ----TC-------------- -------------------- --------------------

                                                                                                                640
C.acrolepis   CCTGTAACTGACAACCCCCG TTAAACCTCACCACCCCTTG TCAATCCCGCCTATATACCA CCGTCGTAAGCTTACCCTAT
C.cinereus    ---A---------------- -------------------- -------------------- --------------------
C.longifili   ---A---------------- -------------------- -------------------- --------------------
C.pectorali   ---A---------------- -------------------- -------------------- --------------------
C.nasutus     ---A---------------- ----------------T--- -------------------- --------------------
C.armatus     ---A---------------- --G----------------- -----T----T--------- --------------------
C.yaquinae    ---A---------------- --G----------T------ -----T----T--------- --------------------
A.macrochir   T--A----------T----- -------------------- ----AT-------------- -------------------C
C.gilberti    T--A----------T----- -------------------- ----AT-------------- -------------------C

                                                                                                                720
C.acrolepis   TGAAGGTTCA/AAAGTAGGC TCTAATAGTAATACTAAAGA CGTCAGGTCGAGGTGTAGCG TATGGGGTGGGAAGAGATGG
C.cinereus    ----------/--------- ----------------G--- -------------------- --------------------
C.longifili   ------A-T-/--------- ----------------G--- -------------------- --------------------
C.pectorali   ----------/--------- ----------------G--- -------------------- --------------------
C.nasutus     --------/-A--------- A---------------G--- -------------------- --------------------
C.armatus     --------ATA--------- C---------------G--- -------------------- C------C------------
C.yaquinae    --------ATA--------- C---------------G--- -------------------- C------C------------
A.macrochir   --------/-A------A-- C-C----------------- -------------------- -----A---------A----
C.gilberti    --------/-A------A-- C-C-----C----------- -------------------- -----A---------A----

                                                                                                                800
C.acrolepis   GCTACATTCCCTAATGAAGA GAATACGAACGGTAATTTGA AAAAATTACACTGAAGGAGG ATTTAGTAGTAAGGAAGGAG
C.cinereus    -------------------- ------------C------- -------------------- --------------------
C.longifili   -------------------- -------------------- -------------------- --------------------
C.pectorali   -------------------- -------------------- -------------------- --------------------
C.nasutus     ---------T---------- ------------C------- -------------------- --------------------
C.armatus     -------------------- ---C--------CG------ ------C------------- -------------A-----A
C.yaquinae    -------------------- ------------CG------ ------C------------- -------------A-----A
A.macrochir   -------------------G ---C-----T----G----- -----C-------------- -------------A-----A
C.gilberti    ------------G-A----G ---C-----T----G----- -----C-------------- -------------A-----A

                                        829
C.acrolepis   CAGAGTGCCCTATTGAAGAT GGCCCTAAT
C.cinereus    ------------C------- ---------
C.longifili   ------------C------- ---------
C.pectorali   -------------------- ---------
C.nasutus     -------------------- ---------
C.armatus     -------------------- ---------
C.yaquinae    ------------C------- ---------
A.macrochir   T----A---T-GC------- ---------
C.gilberti    T--------T-GC------- ---------
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Fig. 4. *continued*

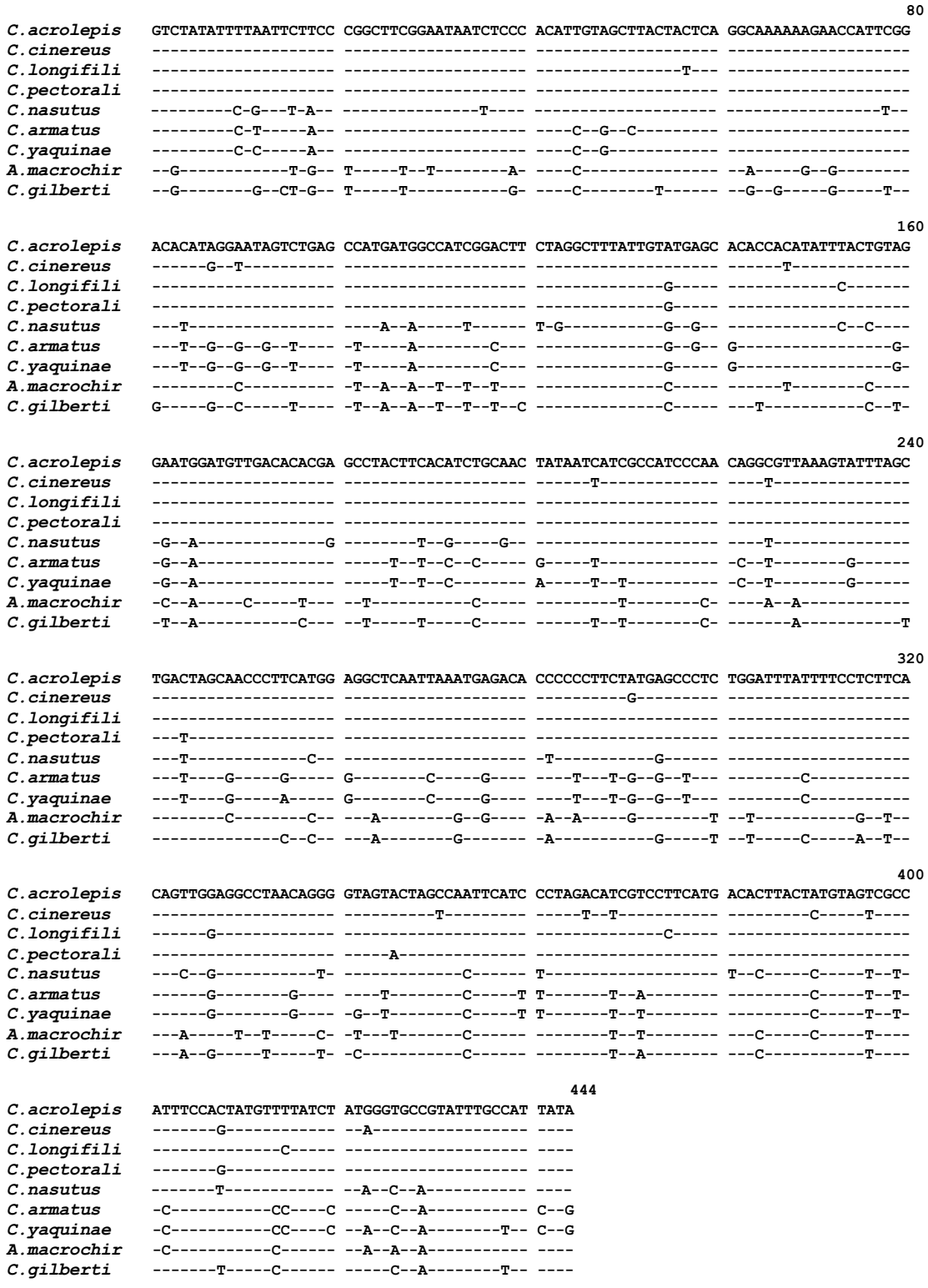

Fig. 5. Comparison of partial nucleotide sequences of the COI gene of *Coryphaenoides* species. A dash indicates an identical nucleotide with that of *C. acrolepis*. The sequences correspond to positions 6167-6611 of the Atlantic cod homolog (Steinar and Ingrid, 1996). The sequences of *Coryphaenoides* species are available from the DDBJ/EMBL/GenBank nucleotide databases with accession numbers AB018233-AB018241.

the transversion of the 12S rRNA gene was not considered to be saturated for these species (Fig. 3 A). Of 829 nucleotides (nt), 114 nt (51 nt in the stem sites and 63 nt in the loop sites) were variable and 79 nt were parsimony-informative (36 nt in stem sites and 43 nt in loop sites). The MP analysis of the 12S rRNA gene produced a most parsimonious length tree with length = 137, consistency index = 0.891, retention index = 0.879, and rescaled consistency index = 0.783 (Fig. 6). This indicates that the phylogenetic positions of the two abyssal species such as *C. armatus* and *C. yaquinae* were separated with a large genetic divergence from the other *Coryphaenoides* species. The phylogenetic trees constructed by the NJ method and the ML method (Ln likelihood = -1874.88) were very similar to the MP tree (Fig. 6).

The COI gene of *Coryphaenoides* species in the region corresponding to positions 6167 to 6611 of the Atlantic cod homolog (Steinar and Ingrid, 1996), was also subjected to phylogenetic analysis. The base composition was A = 25.5%, T = 30.9%, C = 24.4% and G = 19.2%. Nucleotide pairwise distances of the COI gene were greater than those of the 12S rRNA gene and ranged from 0.0090 to 0.1374 (mean = 0.0901) within *Coryphaenoides* and from 0.1374 to 0.1846 (mean = 0.1842) in comparison with the outgroup (Table 3).

Among 444 nucleotide sequences in *Coryphaenoides* species, 117 nucleotide positions were variable and 87 positions were parsimony-informative. The percentage of variable positions in 444 nt of the

Table 2. Pairwise sequence estimates (above diagonal) and number of base substitutions (below diagonal) for 12S rRNA gene

	1	2	3	4	5	6	7	8	9
1. C. armatus		0.0073	0.0571	0.0557	0.0545	0.0570	0.0556	0.0942	0.0885
2 .C. yaquinae	8 (1)		0.0571	0.0584	0.0545	0.0597	0.0556	0.0970	0.0913
3. C. longifilis	45 (7)	45 (7)		0.0049	0.0061	0.0061	0.0286	0.0916	0.0776
4. C. pectoralis	44 (6)	46 (6)	4 (1)		0.0061	0.0037	0.0274	0.0929	0.0789
5. C. cinereus	42 (6)	44 (8)	5 (2)	5 (1)		0.0073	0.0286	0.0902	0.0776
6. C. acrolepis	45 (6)	47 (6)	5 (1)	3 (0)	6 (1)		0.0286	0.0915	0.0775
7. C. nasutus	44 (4)	44 (4)	23 (7)	22 (6)	23 (7)	23 (6)		0.1024	0.0883
8. C. gilberti*	73 (15)	74 (14)	70 (15)	61 (14)	70 (16)	70 (14)	78 (12)		0.0350
9. A. macrochir*	69 (13)	70 (12)	61 (14)	62 (13)	62 (15)	61 (13)	68 (10)	28 (6)	

Above diagonals include pairwise sequence divergenece estimated by the Kimura's two-parameter model. Below diagonals include the number of total base substitutions and that of transversion in parentheses. Asterisks indicate the outgroup species.

Table 3. Pairwise sequence estimates (above diagonal) and number of base substitutions (below diagonal) for COI gene

	1	2	3	4	5	6	7	8	9
1. C. armatus		0.0252	0.1519	0.1525	0.1469	0.1519	0.1488	0.2167	0.2256
2 .C. yaquinae	11 (1)		0.1543	0.1550	0.1438	0.1543	0.1512	0.2141	0.2156
3. C. longifilis	60 (10)	61 (9)		0.0183	0.0442	0.0136	0.0991	0.1822	0.1715
4. C. pectoralis	60 (12)	91 (11)	8 (2)		0.0347	0.0091	0.0969	0.1801	0.1634
5. C. cinereus	58 (12)	57 (11)	19 (2)	15 (2)		0.0300	0.1099	0.1822	0.1540
6. C. acrolepis	60 (10)	61 (9)	6 (0)	4 (2)	13 (2)		0.0991	0.1733	0.1569
7. C. nasutus	59 (9)	60 (8)	41 (3)	40 (5)	45 (5)	41 (3)		0.1664	0.1763
8. C. gilberti*	80 (24)	79 (25)	69 (22)	68 (24)	69 (22)	66 (22)	64 (19)		0.1051
9. A. macrochir*	82 (29)	79 (28)	65 (25)	62 (27)	59 (25)	60 (25)	67 (22)	43 (7)	

Above diagonals include pairwise sequence divergenece estimated by the Kimura's two-parameter model. Below diagonals include the number of total base substitutions and that of transversion in parentheses. Asterisks indicate the outgroup species.

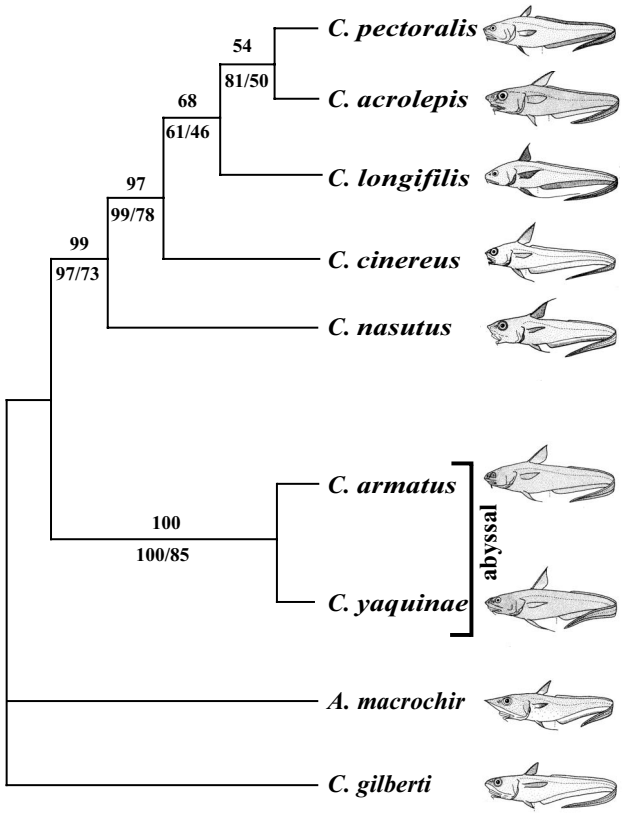

Fig. 6. Phylogenetic tree constructed by the maximum parsimony method based on the 12S rRNA gene of *Coryphaenoides* species. Numbers denote the bootstrap percentages out of 1000 replicates, above branches for MP, left below branches for NJ and right for ML. *A. macrochir* and *C. gilberti* are used as the outgroup. Fish appearances were cited from Nakabo (2000).

COI gene (26.6%) was higher than that in 829 nt of the 12S rRNA gene (13.8%). The TS/TV ratio among *Coryphaenoides* species and outgroup species ranged from 1.0 to 10.0 except for *C. longifilis* and *C. acrolepis* having TV = 0. The TS/TV plot for the COI gene suggested that transitions at the third codon position among the ingroup and outgroup had reached saturation (Fig. 3 B), but not at the first codon position. Of 117 variable nucleotide positions, first and third codon positions accounted for 6.8% and 93.2%, respectively. The MP analysis of the COI gene showed three most parsimonious trees with length = 189, consistency index = 0.778, retention index = 0.722, rescaled consistency index = 0.561, indicating differences among three species, *C. pectoralis*, *C. longifilis* and *C. acrolepis* (Fig. 7 A).

The phylogenetic tree constructed by the NJ method was similar to that by the MP method except that the MP method placed *C. nasutus* in the most basal position of *Coryphaenoides* (Fig. 7 B). The tree based on the COI gene by the NJ method was very similar to that of 12S rRNA gene. The phylogenetic tree constructed by the ML method (Ln likelihood = -1485.98) was similar to one of three

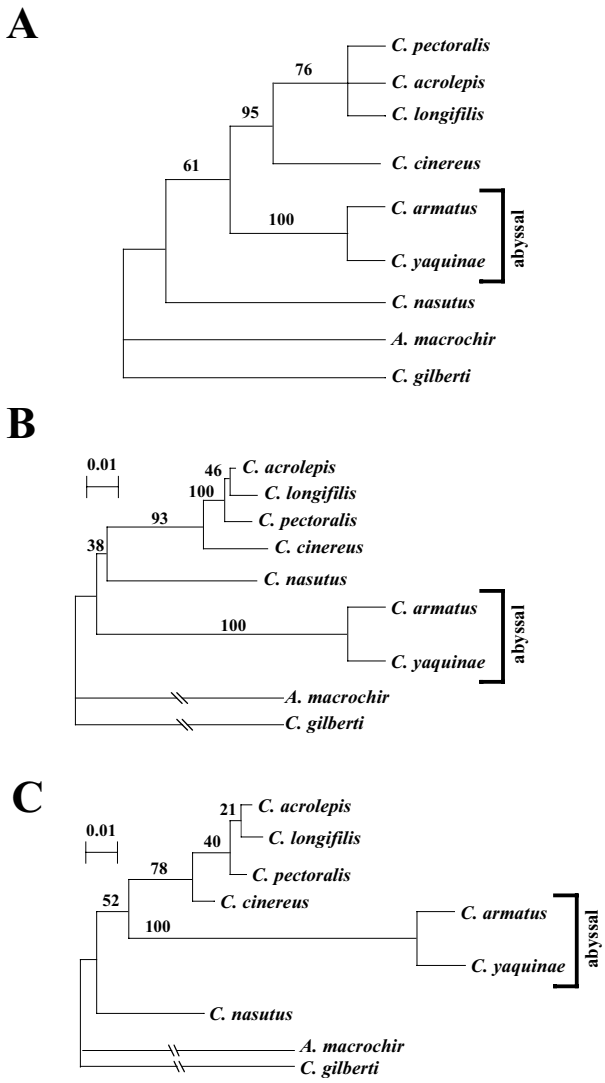

Fig. 7. Phylogenetic trees constructed by maximum parsimony method (A), neighbor joining method (B) and maximum likelihood method (C) based on the COI gene sequences of *Coryphaenoides* species. Numbers on internal branches denotes the bootstrap percentage out of 1000 replicates. Scales indicate the evolutionary distances of the base substitution per site, estimated by the Kimura's two-parameter method. *A. macrochir* and *C. gilberti* are used as the outgroup.

trees by the MP method (Fig. 7 C). Because of the difference in the position of *C. nasutus* among three phylogenetic trees and low bootstrap values for the clade for *C. nasutus* based on the COI gene, the author attempted to reconstruct these phylogenetic tree from data consisting of 1273 bp by combining the sequences of the 12S rRNA (829 bp) and COI (444 bp) genes referring to the previous studies which combined sequence data from different regions of mtDNA (Dutton *et al*., 1996; Harasewych *et al*., 1997; Krajewski *et al*., 1997; Vogler and Welsh, 1997). Fig. 8 shows phylogenetic trees constructed by the MP, NJ and ML methods using combined data of the 12S rRNA and COI genes. The MP method with the combined data produced a most parsimonious tree with length = 329, consistency index = 0.818, retention index = 0.782, rescaled consistency index = 0.639. The trees by the NJ and ML methods (Ln likelihood = -3426.05) with the combined data were the same as constructed by the MP method. The phylogenetic tree based on the combined data was also the same as that based on the 12S rRNA gene. It was noted that the bootstrap values in both the MP and NJ methods based on the combined data were over 60% for every clade and higher than those only on the 12S rRNA gene. Therefore, the phylogenetic tree based on combined data from 12S rRNA and COI genes is considered to be most reliable for the taxonomic position of each *Coryphaenoides* species. Two species such as *C. armatus* and *C. yaquinae*, which are known as abyssal species, were placed at the most basal position in *Coryphaenoides* species, indicating that abyssal species spread into the deep sea earlier than the radiation of other *Coryphaenoides*. The trees imply that five non-abyssal species hereafter diverged successively in the order of *C. nasutus*, *C. cinereus*, *C. longifilis*, *C. acrolepis* and *C. pectorolis*.

Discussion

The present study clearly demonstrated reliable molecular phylogenetic trees of seven *Coryphaenoides* species based on mtDNA analysis and their radiation process into the deep sea. The author's finding proposes new taxonomic positions of *Coryphaenoides* species, while significantly differ from previous taxonic relationships based on morphological (Iwamoto and Sazonov, 1988) and biochemical (Wilson *et al*., 1991; Wilson, 1994) characters. One previous result defining the subgenera was not complete (Iwamoto and Sazonov, 1988). Of *Coryphaenoides* species used in this study, *C. nasutus*, *C. cinereus*, and *C. acrolepis* were included in the subgenus *Coryphaenoides*, whereas *C. armatus*, *C. yaquinae*, *C. pectoralis* and *C. longifilis* were in the subgenus *Nematonurus*.

Wilson (1994) suggested by biochemical studies on LDH isoenzymes that *Coryphaenoides* species diverged on upper continental slope and subsequently spread into the abyssal ocean. However, the phylogenetic trees in the present study placed the abyssal species such *C. armatus* and *C. yaquinae* at the most basal position. This result indicates that the abyssal group separated from non-abyssal one on the upper continental slopes before the radiation of non-abyssal species, and that nonabyssal species thereafter were diverged on the upper slopes. Miya and Nishida (1996) reconstructed the phylogenetic tree of the midwater deep-sea fish genus *Cyclothone* based on mtDNA, showing that *Cyclothone* communities in different localities are of polyphyletic assemblages. The molecular phylogenetic tree of *Cyclothone* placed bathypelagic *Cyclothone* species such as C. obscura and C. parapallida at the most basal position, as well as the tree of *Coryphaenoides* found in this study. It has been believed that new species appear when the gene flow between different populations is interrupted (France and Kocher, 1996). The deep-sea environment is considered to enhance the speciation process by forming a barrier of the gene flow even in the absence of absolute geographic isolation. Hydrostatic pressures have various effects on biochemical and physiological processes (Siebenaller and Somero, 1978, 1989; Siebenaller, 1991; Childress and Thuesen, 1995; Gibbs, 1995; Siebenaller and Murray, 1995). Such marine environment would be one of important factors in the speciation process. The present molecular phylogenetic tree indicates that abyssal *Coryphaenoides* species diverged from non-abyssal species as the first event of *Coryphaenoides*

speciation. The tree also suggests that a new function, which enables species to adapt to high hydrostatic pressures, was provided to proteins by a very few amino acid substitutions rather than by gradual accumulation of successive mutations.

The present phylogenetic trees of *Coryphaenoides* showed that three species, *C. pectoralis*, *C. longifilis* and *C. acrolepis*, are very closely related with their clades having relatively low bootstrap values. The taxonomic position of *C. pectoralis* has long been disputed and this species was recently proposed to be placed in a separate genus Albatrossia (Iwamoto and Sazonov, 1988). However, both NJ and MP analyses of the mitochondrial 12S rRNA and COI genes in the present study indicated that the taxonomic position of *C. pectoralis* is placed within the genus *Coryphaenoides* as in the case of the result of isoenzyme analysis (Wilson *et al.*, 1991; Wilson, 1994). The low bootstrap values were attributed to the small sequence divergence among the three species, 0.0049-0.0061 in the 12S rRNA gene and 0.0180-0.0135 in the COI gene. In order to ascertain the relationship among these species, the analysis of the mitochondrial control region would be more suitable because this analysis has been used for previous studies on closely related species and populations due to its high nucleotide substitution rate (Meyer *et al.*, 1990; Fajen and Breden, 1992; Sturmbauer and Meyer, 1992).

The present findings show that abyssal *Coryphaenoides* such as *C. armatus* and *C. yaquinae* diverged from non-abyssal species as the first event of *Coryphaenoides* speciation. This radiation time can be estimated by the mutation rate of mtDNA, although an absolute divergence time from molecular data cannot be calculated because there is no fossil record for *Coryphaenoides*. Although the mutation rate in mtDNA is estimated to be 1–2 %/MY, it is known to be influenced by thermal habitat, generation time, and metabolic rate (Martin and Palumbi, 1993; Rand, 1994). Ectotherms have slower mutation rates than endotherms, and the mutation rate of typical ectotherms is estimated to be 0.3–0.7%/MY (Kocher *et al.*, 1989; Martin *et al.*, 1992; Martin and Palumbi, 1993; Rand, 1994). The estimated mutation rate for TV (0.14%/MY) in rRNA genes is known to be about the same both in endotherms and ectotherms (Kraus and Miyamoto, 1991; Caconne *et al.*, 1994; Ritchie *et al.*, 1996). Since the average percentage of sequence divergence only in TV between the abyssal and non-abyssal *Coryphaenoides* was 0.724%, the radiation time between the two groups was calculated to be 5.2 million years ago (Fig. 8). In the case of the COI gene, only the TV rate is applicable to the estimation of the radiation time between the abyssal and non-abyssal groups because TS substitutions in this COI gene were saturated and TV was considered to be accumulating approximately linearly with time (Miyamoto and Boyle, 1989). Since the average percentage of sequence divergence in only TV of the COI gene between the abyssal and non-abyssal *Coryphaenoides* was 2.27%, the radiation time was estimated to be approximately 3.2-7.6 million years ago (Fig. 8). Many fossil deep-sea fish found in the stratum between Miocene and Pliocene times have the same morphology as that of the present deep-sea fish, suggesting the occurrence of the present deep-sea fish in these times (Uyeno, 1967, 1980).

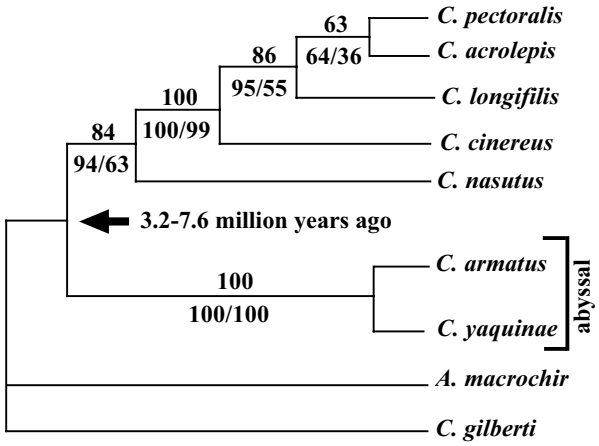

Fig. 8. Phylogenetic tree constructed by the maximum parsimony method by combination data of the 12S rRNA and the COI genes of *Coryphaenoides* species. Numbers denote the bootstrap percentage out of 1000 replicates, above branches for MP, left below branches for NJ and right for ML. *A. macrochir* and *C. gilberti* are used as the outgroup. An arrow indicates the point of the radiation between abyssal and non-abyssal *Coryphaenoides* species, which was estimated to have occured approximately 3.2-7.6 million years ago (see text).

The present study also showed that *Coryphaenoides* arose between Miocene and Pliocene times, about 3.2-7.6 million years ago.

Chapter 2. Characterization and structure of α-skeletal actin of the deep-sea fish

In comparison with studies on thermal biology (Somero, 1995; Hochachka and Somero 2002; Watabe, 2002), those dealing with the effects of pressures on biological functions had been poorly carried out. The development of instruments, however, has accelerated the studies in that area (Mozhaev *et al*., 1996). Hydrostatic pressures act on the volume changes along biological processes. There are a number of reviews dealing with the effects of hydrostatic pressures (Heremans, 1982; Jaenicke, 1983; Weber and Drickamer, 1983; Silva and Weber, 1993; Mozhaev *et al*., 1996). The pressure sensitivity of chemical reactions is dependent upon the magnitude of the volume change associated with the equilibrium constant (K) or rate constant (k), according to the following:

$$\delta \ln K / \delta P = -\delta V / RT$$

$$\delta \ln k / \delta P = -\delta V\# / RT$$

where P = pressure, R = the gas constant, T = the absolute temperature and δV = the difference in volume between the final and initial state at the system at equilibrium and $\delta V\#$ = the difference in volume between the transition and ground states. The response of a reaction or step in a reaction to an increase in pressure is governed by the LeChatelier's principle. If a reaction step proceeds with an increase in volume, the increased pressure will inhibit that step. On the other hand, processes accompanied with a decrease in volume will be enhanced by an increase in pressure.

Section 1. Characterization of the deep-sea fish α-actin under high pressures

Actin has a function in polymerization of G-actin to F-actin in neutral salts. While the volume is increased following polymerization, the reaction is strongly perturbed by high pressures (Ikkai and Ooi, 1966). It is noteworthy that δV, which is associated with polymerization of α-actin in the preparations from abyssal fish *C. armatus*, was much smaller, which is advantageous for a deep-sea habitat, than that from non-abyssal fish *C. acrolepis* (Swezey and Somero, 1985). Actin is a highly conserved protein found in eukaryotic cells; α-actin in carp and that in rat have 99.4% homology at the amino acid sequence level (Collins and Elzinga, 1975; Watabe *et al*., 1995). It is therefore surprising that differences in δV with polymerization of this highly conserved protein have been found between two species of *Corypanenoides* that inhabit different niches.

In this section, the author investigated the polymerization kinetics at high pressures of α-skeletal actin from two abyssal species (*C. armatus* and *C. yaquinae*), non-abyssal species (*C. acrolepis*), carp and chicken.

Materials and Methods

Materials

C. acrolepis specimens (habitat depth 180-2000m) were collected using large deep-sea pots by R/V Soyo-maru of the National Research Institute of Fisheries Science. Sampling locations were 41-40.20' N and 142-57.40' E at the depth of 180 m. *C. armatus* (habitat depth 2700-5000m) and *C. yaquinae* (habitat depth 4000-6400m) were collected as described in Chapter 1. Non-abyssal species, *C. acrolepis*, was selected as a sample fish based on the molecular phylogenetic tree in Chapter 1 and on the amino acid sequences in Section 2 in this chapter. Carp and chicken samples were purchased from local suppliers. All samples were stored below -80℃ until use.

Actin

Actin was isolated from the fast skeletal muscle of each species according to Spudich and Watt (1971) and purified by gel-filtration chromatography on a Sephadex G-200 column (2.5cm × 100cm) in G-buffer (0.2mM ATP, 0.2mM $CaCl_2$, 0.5mM β-mercaptoethanol, 2.0mM Tris-HCl pH 7.8) (MacLean-Fletcher and Pollard, 1980). Actins were stored in G-buffer at 4℃ after purification and used within 3 days. Mg^{2+}-G-actin converted

from Ca^{2+}-G-actin as described previously (Chen and Rubenstein, 1995; Pollard, 1986) was used immediately after the conversion. The concentration of G-actin was determined spectrophotometrically using an absorption coefficient of 0.63mL/mg at 290nm (Houk and Ue, 1974).

Polymerization, critical concentration and δV in assembly of G-actin into F-actin

Polymerization of Mg^{2+}-G-actin was initiated by adding KCl and $MgCl_2$ to a final concentration of 50 and 2 mM, respectively. Polymerization was monitored by light scattering at 4℃, with excitation and emission wavelengths both set at 400nm in a high-pressure cell with a high-pressure pump (PCI-400 cell and TP-500 pump Teramecs) (Fig. 9). The change in light scattering was recorded as a function of time. The critical concentration was determined as described previously (Tobacman *et al.*, 1983; Chen *et al.*, 1993). The volume change (δV) in assembly of G-actin into F-actin was calculated by the method of Swezey and Somero (1985).

Results

Actin polymerization and volume change

The author measured the polymerization kinetics of α-actin isolated from each species as a function of pressure at various actin concentrations by monitoring light scattering assay. Actin prepared from all species except carp underwent polymerization up to 60MPa, the maximum pressure in the present study. However, carp actin polymerized only at pressures of 20 MPa or less. The half-time to steady state in polymerization and critical concentrations were determined from the polymerization kinetics (Fig. 10). The half-times for all actins increased with high pressures, and at atmospheric pressure the polymerization half-time was shortest for carp actin. The half-times for chicken and non-abyssal actin species increased markedly above 20 MPa, and were about 5.6-and 7.3-fold longer, respectively, at 60 Mpa than those at atmospheric pressure (Fig. 10 A). By contrast, the half-time of actin polymerization for the two abyssal species had increased only about 2.7-fold at 60 MPa in the same comparison as above.

The critical concentrations of actin also increased with high pressure for each species (Fig. 10 B). The critical concentrations of the two abyssal actins were higher than those of other species at 20MPa and lower pressures, and increased slightly from 0.1 to 60MPa.

δV associated with polymerization at each pressure was determined from the respective critical concentrations (Fig. 10 C). The two abyssal actin species had a much smaller δV at each pressure than did the other species, in agreement with previous reports (Swezey and Somero, 1985). δV of actins from chicken and non-abyssal species

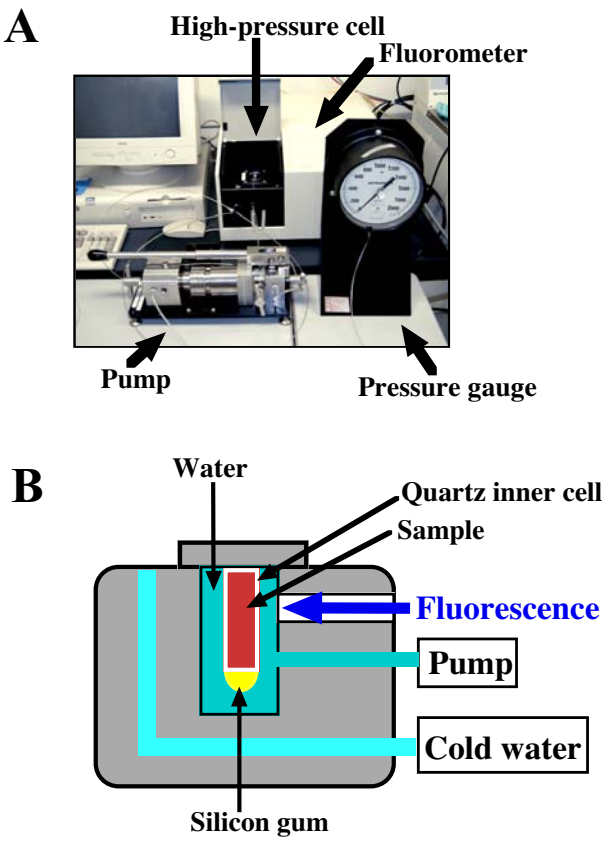

Fig. 9. High-pressure measurement system. (A) Component of high-pressure measurement system. A high-pressure cell is set in a fluorometer. High-pressure is generated with a hand pump. (B) A high-pressure cell. A sample is put in the quartz inner cell. High-pressure generated with the pump is conveyed by the water medium. The water medium pushes up the silicon gum and the sample in the inner cell is compressed. The whole cell is cooled with cold water.

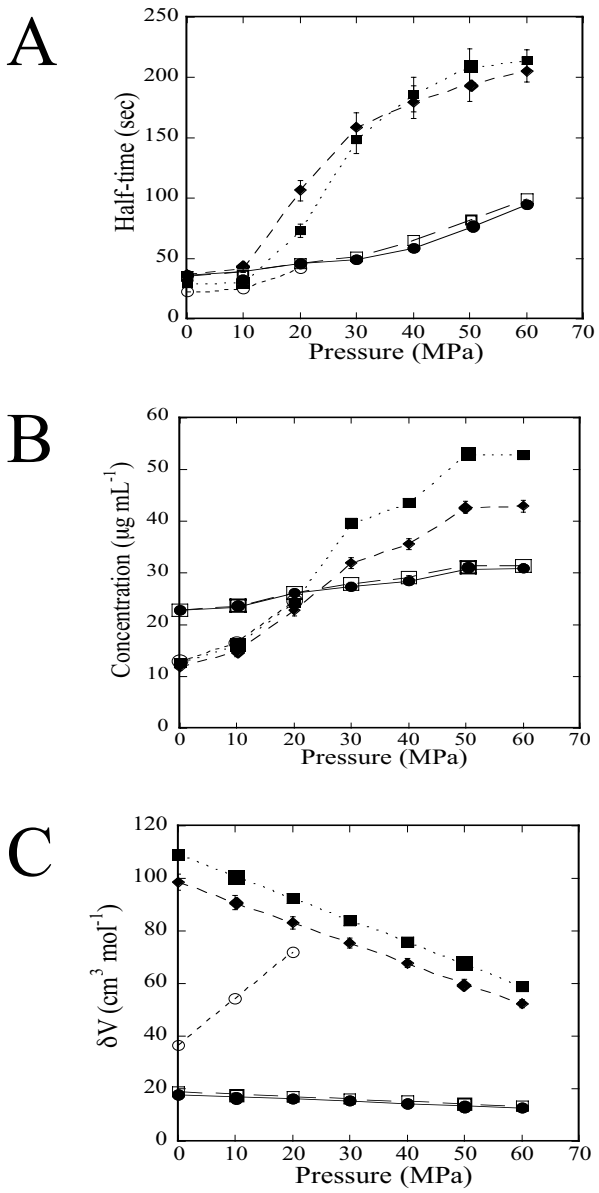

Fig. 10. Effects of pressure on actin polymerization. (A)Half-time to steady state in polymerization of actin. (B)Critical concentration of actin at various pressures. (C)Volume change(δV)associated with polymerization of actin at various pressures. Symbols are as follows: *C. yaquinae*(●); *C. armatus* (□); Chicken(◆); carp(○); C. acrolepis(■). Values are means ± S.D. of four independent experiments. Errors for several experiments are too small to show error bars.

decreased with the increase of pressure, whereas those from the abyssal species showed little variation.

These observations indicate that the coefficient of compressibility for F-actin was larger than that for G-actin for chicken and non-abyssal species, and that the space produced by actin-actin interactions was reduced by high pressures. Clearly, there was no such reduction in space for actins from the abyssal species. Unexpectedly, δV of carp actin, unlike those of other species, increased with the increase of pressure, indicating that the coefficient of compressibility for G-actin was larger than that for F-actin from carp. In other words, carp G-actin was softer than those of other species. Thus, this would explain why carp actin was able to polymerize at pressures of only 20 MPa or less.

Section 2. Molecular cloning of α-actin from the deep-sea fish *Coryphaenoides*

In Section 1 of this chapter, the author demonstrated that α-actin prepared from two abyssal *Coryphaenoides* species are more pressure insenstive and have smaller δV of assembly than those from non-abyssal *Coryphaenoides* species, carp and chicken. Actin is a highly conserved protein; the α-actin in carp and that in rat have 99.4% homology at the amino acid sequence level (Collins and Elzinga, 1975; Watabe *et al.*, 1995). This high homology suggests that a few amino acid substitutions would cause much smaller δV of α-actin from the deep-sea fish.

The objective of this section is to isolate α-actin cDNA clones from the fast skeletal muscle of abyssal and non-abyssal *Coryphaenoides* and identify the amino acid residues making α-actin of the abyssal species adaptive to high hydrostatic pressure.

Materials and Methods

Materials

Samples were collected as described in Chapter 1 and stored frozen after collection. *C. acrolepis* and *C. cinereus* were selected as non-abyssal species based on the results of the molecular phylogenetic analysis in Chapter 1.

Isolation of α-actin cDNA from *C. acrolepis*, *C. armatus* and *C. yaquinae*

Total RNA was extracted from the dorsal fast

skeletal muscles of *C. acrolepis*, *C. armatus* and *C. yaquinae* with RNA extract solution according to the manufacturer's protocol (Isogen; Nippon Gene). Poly (A)+mRNA was isolated from the total RNAs using Oligotex-dT30 <Super>(Takara). About 5g of poly (A)+ mRNA was used to synthesize double-stranded cDNA with a cDNA synthesis kit (Pharmacia Biotech) with the Not-1 d(T) 18primer. The skeletal muscle cDNA libraries were constructed using the synthesized cDNAs and a phage vector λ ZAPII according to the manufacturer's instructions (Stratagene). The α-actin probes were prepared by polymerase chain reaction(PCR). The 5' primer was synthesized referring to the DNA sequences of α-actins from various animals, and corresponded to positions from 372 to 392 of carp α-actin cDNA (Watabe, 1995). The nucleotide sequence of the PCR primers were 5'-CATGTTTGAGACCTTCA ACGT-3' for 5' primer-1 and 5'-TGGAAGAATTCG CGGCCGCA-3' (Not-1 primer) for 3' primer. The conditions of PCR amplification were 30cycles of 94℃ for 30sec, 5℃ for 30sec, and 72℃ for 2min, using a DNA Thermal Cycler Model 2400 (Perkin Elmer). The reaction mixture (100μL) contained 2mM $MgCl_2$, 200mM dNTP, 100pmol of each primer, 2.5units of Taq DNA polymerase, and 50 ng of the cDNA. From these PCR amplifications, the author obtained two PCR products differing in the length from each other. The nucleotide sequences of these PCR products subcloned in a T-vector (Novagen) were homologous to that of the carp α-actin cDNA (Watabe *et al.*, 1995). Therefore, the author synthesized two specific primers that corresponded to the region of 1141 to 1160 nt of *C. acrolepis* α -actin cDNA (Fig. 11), 5' primer-2: 5'-AACATTTG TCTCCATCATTT-3 for α-actin 1, and 5' primer-3: 5'-CACCCAGCGTCTGCTCTCAG-3' for α-actin 2 (Fig. 11), according to these 3'-noncoding regions. PCR amplification was performed under the same conditions described above with Not-1 primer plus 5' primer-2 or 5' primer-3 and using the cDNA libraries. These DNA fragments were labeled with a DIG DNA labeling kit according to the manufacturer's instructions (Boehringer Mannheim) and used as the probes for screening the cDNA library and Northern blot analysis. DNA sequencing was performed with Dye Deoxy terminator cycle sequencing kit using a Model 373A DNA Sequencer (Applied Biosystems).

Isolation of α-actin cDNA from *C. cinereus*

To isolate α-actin cDNAs from *C. cinereus*, reverse transcription (RT)-PCR was performed with sense primers that were synthesized referring to nucleotide sequences in the region of -30 to -11 of the two α-actin cDNA sequences of *C. acrolepis*. The 5' primer-4 for α-actin 1 of 5'-AGCCGCAGACACTC ACCTAA-3' or 5' primer-5 for α-actin 2 of 5'-CCGG AGCTACCAACTGAATA-3' (Fig. 11) was used for PCR with the Not-1 primer as the antisense primer encoding the 3'-end. Single-strand cDNA, which involved the Not-1 primer sequence at the 5'-end, was synthesized from 5μg of total RNA which had been extracted from the dorsal fast skeletal muscle of *C. cinereus* using the T-primed First-strand kit with the Not-1 d $(T)_{18}$ primer (Pharmacia Biotech). One-fourth of the resultant cDNA was used as a template for PCR, the reaction mixture of which (l00 μL)contained 2mM $MgCl_2$, 200mM dNTP, 100pmol of each primer and 2.5units of Taq DNA polymerase. The conditions for PCR were 40 cycles of 94℃ for 1 min, 60℃ for 1 min, and 72℃ for 2 min, using a DNA Thermal Cycler Model 2400 (Perkin Elmer). The two PCR products were subcloned into a T-vector (Novagen). DNA sequencing was performed with Dye Deoxy terminator cycle sequencing kit using a Model 373A DNA Sequencer (Applied Biosystems).

Northern blot analysis

Total RNA was extracted from the dorsal fast skeletal muscle of *C. acrolepis*, and Northern blot analysis was performed using the total RNA (15 μg per lane). RNA was size-fractionated on a 1.2% agarose gel containing 50% formamide by electrophoresis, and then transferred to a nylon membrane (Amersham Bioscience). The probes that had been used for screening of cDNA library were used as the hybridization probes. Hybridization and detection were performed according to the manufacturer's instructions (Boehringer Mannheim).

```
            -49
C.acrolepis-1   TTGGGTCTT TCTCTCTCCA AGCCGCAGAC ACTCACCTAA GAAAGCCATC
C.acrolepis-2a    TTAC... GT.....GAT CC..CGGAG. TAC..A..G. AT...T..A.
C.cinereus-1                                                G........
C.cinereus-2a                                                 ..T..A.
C.armatus-2a                       T CC..CGGAG. TAC..A..G. AT...T..A.
C.armatus-2b                    ..... .......... .......... .G.....T..
C.yaquinae-2a                      T CC..CGGAG. TAC..A..G. AT...T..A.
C.yaquinae-2b                   ..... .......... .......... .G.....T..

              START                                                    50
C.acrolepis-1   ATGTGTGACG ATGAGGAGAC TACCGCCCTT GTGTGCGACA ACGGCTCCGG
C.acrolepis-2a  .......... .C..A..... C........C .......... ..........
C.cinereus-1    .......... .......... .......... .......... ..........
C.cinereus-2a   .......... .C..A..... C........C .......... ..........
C.armatus-2a    .......... .C..A..... C........C .......... ..........
C.armatus-2b    .......... .......... .......... .......... ..........
C.yaquinae-2a   .......... .C..A..... C........C .......... ..........
C.yaquinae-2b   .......... .......... .......... .......... ..........

                                                                    100
C.acrolepis-1   CCTGGTGAAG GCTGGGTTCG CCGGAGACGA TGCCCCCAGG GCTGTCTTCC
C.acrolepis-2a  ...C...... .....C.... .......... C..A...... ..C.......
C.cinereus-1    .......... .......... .......... .......... ..........
C.cinereus-2a   ...C...... .....C.... .......... C..A...... ..C.......
C.armatus-2a    ...C...... .....C.... .......... C..A...... ..C.......
C.armatus-2b    ...C...... .......... .......T.. C..G...... ..........
C.yaquinae-2a   ...C...... .....C.... .......... C..A...... ..C.......
C.yaquinae-2b   ...C...... .......... .......T.. C..G...... ..........

                                                                    150
C.acrolepis-1  CCTCCATCGT CGGCCGCCCC CGTCACCAGG GTGTCATGGT CGGTATGGGT
C.acrolepis-2a .A........ .........T .......... .C........ G..C.....G
C.cinereus-1   .......... .......... .......... .......... ..........
C.cinereus-2a  .A........ .........T .......... .C........ G..C.....G
C.armatus-2a   .A........ .........T .......... .C........ G..C.....G
C.armatus-2b   .A........ .......... .......... .......... A..C.....G
C.yaquinae-2a  .A........ .........T .......... .C........ G..C.....G
C.yaquinae-2b  .A........ .......... .......... .......... A..C.....G

                                                                    200
C.acrolepis-1  CAGAAAGACT CCTACGTCGG CGACGAGGCC CAGAGCAAGA GAGGTATCTT
C.acrolepis-2a .....G.... .......G.. .......... .......... ........C.
C.cinereus-1   .....G.... .......... .......... .......... ..........
C.cinereus-2a  .....G.... .......G.. .......... .......... .G......C.
C.armatus-2a   .....G.... .......G.. .......... .......... .G..C...C.
C.armatus-2b   .....G.... ......CG.. .......... .......... .G......C.
C.yaquinae-2a  .....G.... .......G.. .......... .......... .G..C...C.
C.yaquinae-2b  .....G.... .......G.. .......... ........A. .G......C.

                                                                    250
C.acrolepis-1  GACTCTTAAG TACCCCATTG AGCACGGCAT CATCACCAAC TGGGACGACA
C.acrolepis-2a ...C..C... ........C. .......... .......... ..........
C.cinereus-1   .......... .......... .......... .......... ..........
C.cinereus-2a  ...C..C... ........C. .......... .......... ..........
C.armatus-2a   ...C..C... ........C. .......... .......... ..........
C.armatus-2b   ...C..C... .......... .......... .........T ..........
C.yaquinae-2a  ...C..C... ........C. .......... .......... ..........
C.yaquinae-2b  ...C.CC... .......... .......... .........T ..........
```

Fig. 11. Comparison of cDNA nucleotide sequences encoding α-actin 1, α-actin 2a and α-actin 2b. The dot indicates an identical nucleotide with that of *C. acrolepis* α-actin 1. 5' primer-2, 3, 5' primer-4, 5 and polyadenylation signals are indicated by red, blue and boldfaced letters, respectively. These sequences are available from the DDBJ/EMBL/GenBank databases. The accession numbers are AB021649, AB021650, AB021651, AB021652, AB086240, AB086241, AB086242, and AB086242 from the top.

```
                                                                      300
C.acrolepis-1   TGGAGAAGAT CTGGCACCAC ACCTTCTACA ATGAGCTGCG CGTGGCCCCC
C.acrolepis-2a .......... .......... .......... .C........ T.........
C.cinereus-1    .......... .......... .......... .......... ..........
C.cinereus-2a   .......... .......... .......... .C........ T.........
C.armatus-2a    .......... .......... .......... .C........ T.........
C.armatus-2b    .......... .......... .......... .C........ ..........
C.yaquinae-2a   .......... .......... .......... .C........ T.........
C.yaquinae-2b   .......... .......... .......... .C........ ..........

                                                                      350
C.acrolepis-1   GAGGAGCACC CCACCCTGCT CACTGAGGCC CCCCTGAACC CCAAGGCTAA
C.acrolepis-2a .......... .......... ...C...... .....C.... .......C..
C.cinereus-1    .......... .......... .......... .......... ..........
C.cinereus-2a   .......... .......... ...C...... .....T.... .......C..
C.armatus-2a    .......... .....T.... ...C...... .....C.... .......C..
C.armatus-2b    .......... .......... .......... .....C.... ..........
C.yaquinae-2a   .......... .......... ...C...... .....C.... .......C..
C.yaquinae-2b   .......... .......... .......... .....C.... ..........

                                                                      400
C.acrolepis-1   CCGTGAAAAG ATGACCCAGA TCATGTTTGA GACCTTCAAC GTCCCCGCCA
C.acrolepis-2a .A.G..G... .......... .......C.. .......... ..G.......
C.cinereus-1    .......... .......... .......... .......... ..........
C.cinereus-2a   .A.G..G... .......... .......C.. .......... ..G.......
C.armatus-2a    .A.G..G... .......... .......C.. .......... ..G.......
C.armatus-2b    .A....G... .....A.... .......... .......... ..G.......
C.yaquinae-2a   .A.G..G... .......... .......C.. .......... ..G.......
C.yaquinae-2b   .A....G... .....A.... .......... .......... ..G.......

                                                                      450
C.acrolepis-1   TGTATGTGGC CATCCAGGCT GTGCTGTCCC TGTACGCTTC CGGCCGTACC
C.acrolepis-2a ....C..... .........C .......... .......C.. ..........
C.cinereus-1    .......... .......... .......... .......... ..........
C.cinereus-2a   ....C..... .........C .......... .......C.. ..........
C.armatus-2a    ....C..... .........C .......... .......C.. ..........
C.armatus-2b    .......... ....A....C .......... .......... ......C...
C.yaquinae-2a   ....C..... .........C .......... .......C.. ..........
C.yaquinae-2b   .......C.. ....A....C .......... .......... ...A..C...

                                                                      500
C.acrolepis-1   ACCGGTATTG TGCTGGATGC TGGTGATGGT GTCACCCACA ACGTCCCAGT
C.acrolepis-2a ..T.....C. .......CT. C.....C... ..G.......  ....G..G..
C.cinereus-1    .......... .......... .......... .......... ..........
C.cinereus-2a   ..T.....C. .......CT. C.....C... ..G.......  ....G..G..
C.armatus-2a    ..T.....C. .......CT. C.....C... ..G.......  ....G..G..
C.armatus-2b    ..T.....C. .......CT. C..G..C... ..G.....T.  ....G..G..
C.yaquinae-2a   ..T.....C. .......CT. C.....C... ..G.......  ....G..G..
C.yaquinae-2b   ..A.....C. .......CT. C..G..C... ..G.....T.  .......C..

                                                                      550
C.acrolepis-1   GTATGAGGGT TACGCCCTGC CCCACGCCAT CATGCGTCTT GACCTGGCTG
C.acrolepis-2a .........C .....G.... .......... ......C..G .........C.
C.cinereus-1    .......... .......... .......... .......... ..........
C.cinereus-2a   .........C .....G.... .......... ......C..G .........C.
C.armatus-2a    ...C.....C .....G.... .......... ......C..G .........C.
C.armatus-2b    ...C...... .....T.... .......... ....A.G..G ...T....C.
C.yaquinae-2a   ...C.....C .....G.... .......... ......C..G .........C.
C.yaquinae-2b   A..C.....C .....T.... .......... ....A.GT.G ...T....C.

                                                                      600
C.acrolepis-1   GTCGCGACCT GACCGACTAC CTGATGAAGA TCCTGACCGA GCGTGGCTAC
C.acrolepis-2a .C........ C......... ..C....... ....C..... ...C..A...
C.cinereus-1    .......... .......... .......... .......... ..........
C.cinereus-2a   .C........ C......... ..C....... ....C..... ...C..A...
C.armatus-2a    .C........ C......... ..C....... ....C..... ...C..A...
C.armatus-2b    .......... C......... ..C....... ....C..... .A.G..G...
C.yaquinae-2a   .C........ C......... ..C....... ....C..... ...C..A...
C.yaquinae-2b   ....G..... C..A...... ..C....... .......... .A.G......
```

Fig. 11. *continued*

```
                                                                                                650
C.acrolepis-1  TCTTTCGTTA CCACCGCCGA GCGTGAGATC GTGCGCGACA TCAAGGAGAA
C.acrolepis-2a ..C.....C. .......... .......... .......... ..........
C.cinereus-1   .......... .......... .......... .......... ..........
C.cinereus-2a  ..C.....C. .......... .......... .......... .......A..
C.armatus-2a   ..C.....C. .......... ...G...... .......... ..........
C.armatus-2b   ........C. .......... .......... .......... ..........
C.yaquinae-2a  ..C.....C. .......... ...G...... .......... ..........
C.yaquinae-2b  AG......C. .......... .......... .......... ..........

                                                                                                700
C.acrolepis-1  GCTGTGCTAT GTGGCTCTGG ACTTCGAGAA CGAGATGGCC ACCGCCGCCT
C.acrolepis-2a .........C ..C..C.... .......... .........A ..........
C.cinereus-1   .......... .......... .......... .......... ..........
C.cinereus-2a  .........C ..C..C.... .......... .........A ..........
C.armatus-2a   .........C ..C..C.... .......... .........G ..........
C.armatus-2b   .......... .....G.... .......... .........G ........G.
C.yaquinae-2a  .........C ..C..C.... .......... .........G ..........
C.yaquinae-2b  .......... .....G.... .......... .........G ........T.

                                                                                                750
C.acrolepis-1  CCTCCTCCTC CCTGGAGAAG AGCTACGAGC TTCCCGACGG TCAGGTCATC
C.acrolepis-2a .......... .......... .....T.... .G........ ..........
C.cinereus-1   .......... .........A .......... .......... ..........
C.cinereus-2a  .......... .......... .......... .G........ ..........
C.armatus-2a   .......... .......... .......... .G........ ..........
C.armatus-2b   .......A.. T......... .......... .G........ ..........
C.yaquinae-2a  .......... .......... .......... .G........ ..........
C.yaquinae-2b  .......A.. .......... ..T....... .G........ ..........

                                                                                                800
C.acrolepis-1  ACCATAGGAA ACGAGCGTTT CCGTTGCCCT GAGACCCTCT TCCAGCCTTC
C.acrolepis-2a .....C..C. .......G.. ...C.....C .......... .......C..
C.cinereus-1   .....C.... .......... .......... .......... ..........
C.cinereus-2a  .....C..C. .......C.. ...C.....C .......... .......C..
C.armatus-2a   .....C..C. .......C.. ...C.....C .......... .......C..
C.armatus-2b   ........C. .......C.. ...C...... .......... ..........
C.yaquinae-2a  .....C..C. ....A..C.. ...C.....C .......... .......C..
C.yaquinae-2b  ........C. .......C.. ...C...... .......... ..........

                                                                                                850
C.acrolepis-1  CTTCATCGGA ATGGAGTCCG CCGGTATCCA TGAGACCGCC TACAACAGCA
C.acrolepis-2a  .........C ........T. ....C..... C......... ..........
C.cinereus-1   .......... .......... .......... .......... ..........
C.cinereus-2a  .........C ........T. ....C..... C......... ..........
C.armatus-2a   .........C .......... ....C..... C......... ..........
C.armatus-2b   .........T .......... .......... .......... ..........
C.yaquinae-2a  .........C .......... ....C..... C......... ..........
C.yaquinae-2b  .........T ........G. .......... .......... ..........

                                                                                                900
C.acrolepis-1  TCATGAAGTG CGACATTGAC ATCCGCAAGG ACCTGTACGC CAACAACGTG
C.acrolepis-2a .......... ......C... .......... .......... ..........
C.cinereus-1   .......... .......... .......... .......... ..........
C.cinereus-2a  .......... ......C... .......... .......... ..........
C.armatus-2a   .......... ......C... .......... .......... ..........
C.armatus-2b   .......... ......C... .......... .......... .........T
C.yaquinae-2a  .......... ......C... .......... .......... ..........
C.yaquinae-2b  .......... ......C... .......... .......... ..........

                                                                                                950
C.acrolepis-1  CTCTCCGGTG GTACCACCAT GTACCCTGGT ATTGCTGACC GTATGCAGAA
C.acrolepis-2a ..G.....C. C........ .......... .......... .C........
C.cinereus-1   .......... .......... .......... .......... ..........
C.cinereus-2a  ..G.....C. .C........ .......... ..C....... .C........
C.armatus-2a   ..G.....C. .C........ ......C... ..C....... .C........
C.armatus-2b   ..G....... .......... .......... ..C....... ..........
C.yaquinae-2a  ..G.....C. .C........ ......C... ..C....... .C........
C.yaquinae-2b  ..G....... .......... ......G... ..C..C.... ..........
```

Fig. 11. *continued*

```
                                                                      1000
C.acrolepis-1   GGAGATCACC GCCCTGGCCC CATCCACCAT GAAGATCAAG ATCATTGCTC
C.acrolepis-2a ..........  ........G. .T........ .......... .....C..C.
C.cinereus-1    ..........  .......... .......... .......... ..........
C.cinereus-2a   ..........  ........G. .T........ .......... .....C..C.
C.armatus-2a    ..........  ........G. .T........ .......... .....C..C.
C.armatus-2b    ..........  ........A. .C........ .......... ..........
C.yaquinae-2a   ..........  ........G. .T........ .......... .....C..C.
C.yaquinae-2b   .........A  ........A. .G........ .......... ..........

                                                                      1050
C.acrolepis-1   CCCCCGAGAG GAAGTACTCC GTCTGGATCG GTGGCTCCAT CCTGGCTTCC
C.acrolepis-2a ....A.....  .........T .......... .A........ ......C...
C.cinereus-1    ..........  .......... .......... .......... ..........
C.cinereus-2a   ....A.....  .........T .......... .A........ ......C...
C.armatus-2a    ....A.....  .........T .......... .A........ ......C...
C.armatus-2b    ....A.....  .......... .......... .......... ..........
C.yaquinae-2a   ....A.....  .........T ..G....... .A........ ......C...
C.yaquinae-2b   ....A.....  .......... .......... .......... ..........

                                                                      1100
C.acrolepis-1   CTGTCCACCT TCCAGCAGAT GTGGATCTCC AAGCAGGAGT ACGACGAGGC
C.acrolepis-2a ..........  ..........  .......AG. .......... ..........
C.cinereus-1    ........A.  .......... .......... .......... ..........
C.cinereus-2a   ..........  ..........  .......AG. .......... ..........
C.armatus-2a    ..........  ..........  .......AG. .......... ..........
C.armatus-2b    ..........  ..........  .......... .......... ..........
C.yaquinae-2a   ..........  ..........  .......AG. .......... ..........
C.yaquinae-2b   ..........  ..........  .......... .......... ..........

                                                  STOP                1150
C.acrolepis-1   AGGCCCCAGC ATTGTCCACA GGAAGTGCTT CTAAATCCTC AACATTTGTC
C.acrolepis-2a C......TC.  ..C.......  ..........  .......AC. C..CCAGCGT
C.cinereus-1    ..........  ..........  ..........  .......... ..........
C.cinereus-2a   C......TC.  ..C.......  ..........  .......AC. C..CCAGCGT
C.armatus-2a    C......TC.  ..C.......  ..........  .......AC. C..CCAGCGT
C.armatus-2b    ..........  ..........  ..........  .......AC. CG.CCAGCGT
C.yaquinae-2a   C......TC.  ..C.......  ..........  .......AC. C..CCAGCAT
C.yaquinae-2b   ..........  ..........  ..........  .......AC. CG.CCAGCGT

                                                                      1200
C.acrolepis-1   TCCATCATTT TCGCCATCAC CATCATCACC ACCTGTCACC AGGAGATCGG
C.acrolepis-2a CTGC..TCAG  GACA..A.CA ACCGG.GG.A CGAC.G.G.. .CCCAGGA.C
C.cinereus-1    ..........  ..........  ...T......  ..........  ..........
C.cinereus-2a   CTGC..TCAG  GACA..A.CA ACCGG.GG.A CGAC.G.G.. .CCCAGGA.C
C.armatus-2a    CTGC..TCAG  GACA..A.CA ACCGG.GG.A CGAC.G.G.. .CCCAGGA.C
C.armatus-2b    CTGC..TCAG  GACA..A.CA ACCGG.GG.A CGAC.G.G.. .CCCAGGG.C
C.yaquinae-2a   CTGC..TCAG  GACA..A.CA ACCGG.GG.A CGAC.G.G.. .CCCAGGA.C
C.yaquinae-2b   CTGC..TCAG  GACA..A.CA ACCGG.GG.A CGAC.G.G.. .CCCAGGA.C

                                                                      1250
C.acrolepis-1   GCGAGGAGGA GGACCACCCC ATGCGGCACA GCGACACCCT GCTGCTCATA
C.acrolepis-2a CGCCTAGT.G  AC.TTTTGTT G.TGTTGTTG TT.TTGTGGA TG..G.AG..
C.cinereus-1    ..........  ..........  ..........  ..........  ..........
C.cinereus-2a   CGCCTAGT.G  AC.TTTTGTT G.TGTTGTTG TT.TGGATG. .G.AG.AG.T
C.armatus-2a    CGCCTA.T.G  AC.TTTTGTT G.TGTTGTTG TT.TTGTGGA TG..G.AG..
C.armatus-2b    CGCCTAGT.G  AC.TTTTGTT G.TGTTGTTG TT.TTGTGGA TG..G.AG..
C.yaquinae-2a   CGCCTAGT.G  AC.TTTTGTT G.TGTTGTTG TT.TTGTGGA TG..G.AG..
C.yaquinae-2b   CGCCTAGT.G  AC.TTTTGTT G.TGTTGTTG TT.TGGATG. .G.AG.AG.T

                                                                      1300
C.acrolepis-1   TGCATTTTTT TTTATGATTC TTGAATCTGC ATATCGTACT GGCTGTCTGT
C.acrolepis-2a GTTG..G..G  ..GTG.TAGT GGA.GAAGTG T.TGGTGGTG T.G.CAACCG
C.cinereus-1    ..........  ..........  ..AG......  .....TCT..  ..........
C.cinereus-2a   GTTG..G..G  .GGTG.TGGA AGAGG.G.TT GGTGGTGTGG TCACCGAA.C
C.armatus-2a    GTTG..G..G  ..GTG.TACT GGA.GAAGTG T.TGGTGGTG T.G.CA.C.A
C.armatus-2b    GTTG..G..G  .GGTG.TGGT GA...GG..T T.TGG.CTG. .TTG...ACC
C.yaquinae-2a   GTTG..G..G  ..GTG.TACT GGA.GAAGTG T.TGGTGGTG T.G.CAACCG
C.yaquinae-2b   GTTG..G.GG  .GGTG.TGGA GGAGG.G.TT GGCGGTGTG. TCACCGAA.C
```

Fig. 11. *continued*

```
                                                                      1350
C.acrolepis-1  TGTCTGTGTT GTGCGGAGAA CAAGCTGTGA ACAAACAACA CTGAACCATG
C.acrolepis-2a AAG.GCAAG. ...TA.C.C. A.CCGGAGCG .GC.G.GGGG GGCG.G..G.
C.cinereus-1   .......... ........G. .......... .........G A.........
C.cinereus-2a  GCAAGTGTG. AGCGCA.ACC GG...GAGC. G.GGGGGG.G AGC.GGG.G.
C.armatus-2a   A.CGCAA..G TGTA.CGC.. .CG.AGCGA. CAGCGGGGGG .GAGCAAGGA
C.armatus-2b   GAAGC.CAAG CGTGTACCGC A.CCGGAGCG .AC.CGGGGG GG.GGAGCA.
C.yaquinae-2a  AAG.GCAAG. ...TA.C.C. A.CCGGA.CG CGGGGGGGGG GG.G.G..G.
C.yaquinae-2b  GCAAGCGTG. AGCGCA.CCG G.GCGA.CAG .AC.CGGCGG GC.G.G..GT

                                                                      1400
C.acrolepis-1  CATCTAACCC CCAAAAAATT AATAAAAAAA TCAAAACAAA AAAAAAAAAA
C.acrolepis-2a G.GG.C.G.A .GTG..CGCG TGA.G..CTG .AC.T.TG.T T.TTT.TTG.
C.cinereus-1   ....C..... .......... .......... ATC...AC.. C.........
C.cinereus-2a  TCAGC.CGTG AACGCGTGAA G.ACTGT.C. .ATG.TT.TT T.TTG.TT.T
C.armatus-2a   AG..AGCA.G TG..CGCT.G ..AG...CTG .AC.T.ATG. .T.TTT.TTG
C.armatus-2b   TGAGGTCAG. A.GTG..GAA .CA.C..... .A....ACC. CC........
C.yaquinae-2a  G.AG.C.G.A ..TG..CGCG TGA.G..CTG .AC.T.TG.. T.TTT.TTG.
C.yaquinae-2b  G.GG.C.G.A .GTG..G.AA C.AC.....T AA.....C.C C.........

                                                                      1450
C.acrolepis-1  AAAAA
C.acrolepis-2a TT.TGCAGGC CTACATGGAG GGATTGTTTT GTAAGGACAG GGGCCCGTGG
C.cinereus-1   .....AAAAA A
C.cinereus-2a  GC.GGCCTAC ATGGAGGGAT TGTTTTGTAA GGACAGGGGC CCGTGGCCCC
C.armatus-2a   .TT.TGCAGG CCTACATGGA AGGATGTTTG TTAGAACGGG GGCCCCACGG
C.armatus-2b  .
C.yaquinae-2a  TT.TGCAGGC CTACATGGAG GGATTGTTAG AAAAGGGGCC CACGGCCCCG
C.yaquinae-2b  .

                                                                      1500
C.acrolepis-1
C.acrolepis-2a CCCCGCAGGC GATGAGTTGG CTGTTGGCTT CGAGGTCGAG GAAAACCCCG
C.cinereus-1
C.cinereus-2a  GCAGGCGATG AGTTGGCTGT TGGCTTCGAG GTCGAGGAAA ACCCCGAACA
C.armatus-2a   CCCCGCAGGA GACGAGTTGG CTGTTGGCTT CGAGGTCGAG GAAAACCCCG
C.armatus-2b
C.yaquinae-2a  CAGGAGACGA GTTGGCTGTT GGCTTCGAGG TCGAGGAAAA CCCCGAACAA
C.yaquinae-2b

                                                                      1550
C.acrolepis-1
C.acrolepis-2a AACAAAGAAA TGAAAAAGAC CAACAAAAAA AAAAGAACAA AGCAAATAAA
C.cinereus-1
C.cinereus-2a  AAGAAATGAA AAAGACCAAC AAAAAAAAAA AAGAACAAAG CAAATAAAAT
C.armatus-2a   AACAAAGAAA TGAAAAAGAC CAACAAAAAA AAAAAAGAAC AAAGCAAATA
C.armatus-2b
C.yaquinae-2a  AGAAATGAAA AAGACCAACA AAAAAAAAAA AGAACAAAGC AAATAAAATA
C.yaquinae-2b

                                                            1587
C.acrolepis-1
C.acrolepis-2a ATAATTTATT TTCTAAAAAA AAAAAAAAAA AAAAAA
C.cinereus-1
C.cinereus-2a  AATTTATTTT CAAAAAAAAA AAAAAAAA
C.armatus-2a   AAATAATTTA TTTTCAAAAA AAAAAAAAAA AAAAAAA
C.armatus-2b
C.yaquinae-2a  ATTTATTTTC AAAAAAAAAA AAAAAAAAAA AA
C.yaquinae-2b
```

Fig. 11. *continued*

Quantification of actin isoforms

To determine the ratio of the α-actin isoforms, quantitative RT-PCR and two-dimensional electrophoresis were performed. These analyses were performed four times using total RNAs and actin preparations isolated from four individuals in each species. The conditions for RT-PCR were the same as described above, except for cycle number. The cycle number within a linear range of PCR amplification was determined to be 25 on the basis of the signal intensities of RT-PCR products with sequential cycles. The primers of 5'-ATTGCTGACCGYATGCAGAA-3' and Not-1 d $(T)_{18}$ amplified approximately 480 bp and 680 bp for actin 1 and actin 2b, respectively. RT-PCR products were subjected to 1.5% agarose gel electrophoresis. Two-dimensional electrophoresis was performed with Multiphor II electrophoresis unit in the pH range of 4.0-7.0 or 5.0-6.0 gel (24cm) and on a 12.5% SDS-PAGE gel (Amersham Bioscience). The ratios of actin 2b to actin 2a, or actin 1 to actin 2a were quantified using a computerized image analysis scanner STORM 860 (Amersham Bioscience).

Phylogenetic analysis

A molecular phylogenetic tree was constructed from the deduced amino acid sequences of actin. The DNADIST and NEIGHBOR programs in the PHYLIP version 3.5 program package (Felsenstein, 1995) were used for neighbor-joining (Saitou and Nei, 1987). Bootstrap analyses with 1000 replicates were performed to examine the confidence of nodes within the resultant topology. The GenBank accession numbers of actin nucleotide sequences used in this study were *C. armatus* 2a (AB086240), *C. armatus* 2b (AB086241), *C. yaquinae* 2a (AB086242), *C. yaquinae* 2b (AB086243), *C. acrolepis* 1 (AB021649), *C. acrolepis* 2a (AB021650), *C. cinereus* 1 (AB021651), *C. cinereus* 2a (AB021652); carp, *Cyprinus carpio* (D50025); medaka, *Oryzias latipes* (D87740); fugu 1, *Fugu rubripes* (U38850), fugu 2 (U38958); goldfish, *Carassius auratus* (D50029); tilapia, *Oreochromis mossambicus* (AB037866); zebrafish, *Danio rerio* (AF180887); chum salmon, *Oncorhynchus keta* (AB032464); atlantic salmon, *Salmo salar* (AF304406); chicken, *Gallus gallus* (K02257); human, *Homo sapiens* (M20543); mouse, *Mus musculus* (M12234); rat, *Rattus norvegicus* (V01218); bovine, *Bos taurus* (U02285). medaka α-actin (D89627) was used as the outgroup gene.

Results

cDNA cloning and deduced amino acid sequences

Two α-actin isoforms were cloned from each *Coryphaenoides* species. The nucleotide sequence was determined for the longest insert including the entire coding region of the positive clones from the respective libraries. The RT-PCR strategy was successful in cloning the entire length of the two α-actin cDNAs from *C. cinereus* (Fig. 11).

Molecular phylogenic analysis based on the coding regions of α-actin gene showed that the two types of isoform each from *C. acrolepis* and *C. cinereus* are categorized as actin 1 and actin 2, respectively, previously reported for pufferfish (Fig. 12). On the other hand, two types each from the two abyssal species were all categorized as actin 2. These categorizations were supported by the comparatively high bootstrap value (72%). Subsequently, the isoform with an identical amino acid sequence to that of actin 2 from the non-abyssal species was designated actin 2a, and the others are designated arm actin 2b and yaq actin 2b from *C. armatus* and *C. yaquinae*, respectively. Consequently, the non-abyssal actin 2 was re-designated actin 2a.

The deduced amino acid sequence of α-actins from four *Coryphaenoides* species contained 337 amino acid residues. Since the N-terminal two amino acids, Met-Cys, are known to be processed after translation (Pollard and Cooper, 1986), α-actin consists of 375 amino acids. The amino acid sequences of α-actin 1 from *C. acrolepis* and α-actin 1 from *C. cinereus*, were identical, as were the amino acid sequences of α-actin 2a from the four species. The amino acid sequences of actin 1 and 2a differ by one amino acid residue at position 155, which is Ala-155 in actin 1 and Ser-155 in actin 2a. The sequence of actin 2b differs from that of actin 2a by two amino acids (Q137K and either V54A or L67P) (Table 4). The X-ray crystallography

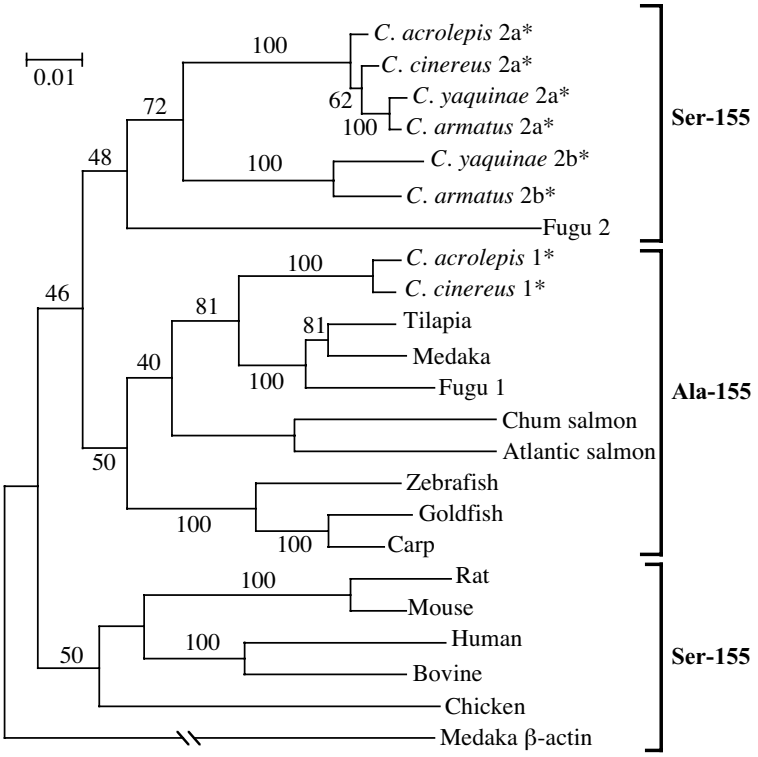

Fig. 12. Molecular phylogentic tree constructed by neighbor-joining method based on nucleotide sequences in the coding regions of actin cDNA from various species. Numbers at internal branches denote the bootstrap percentages of 1000 replicates. The scale indicates the evolutionary distance of the base substitution per site, estimated by the Kimura two-parameter method. The Medaka α-actin gene was used as the outgroup gene. Asterisks indicate the cDNAs that were cloned in this study.

structure of rabbit skeletal muscle α-actin demonstrated that residues 54 and 67 are located in a β-sheet of subdomain 2 (residues 33-69), whereas residues 137 and 155 are located in the Ca^{2+}- and ATP-binding sites, respectively (Kabsch *et al.*, 1990) (Fig. 13).

Northern blot analysis and quantification of actin isoforms

To examine whether these two α-actin isoform mRNAs are expressed in *C. acrolepis*, Northern blot analysis was performed with total RNA that had been extracted from the dorsal skeletal muscle of *C. acrolepis*. Two bands slightly differing on the mobility in the agarose gel were observed (Fig. 14 A). The probes used in the northern blot analysis were derived from 3'-noncoding regions, which considerably differed in nucleotide sequences between the two isoforms. Therefore, the probes were considered to be specific to the respective isoforms. The same result was obtained using total RNA extracted from the dorsal fast skeletal muscle of *C. cinereus*. Two α-actin isoform mRNAs are also expressed in the fast muscle of pufferfish (Venkatesh *et al.*, 1996). The results of Northern blot analysis do not indicate the expression ratio of two isoforms due to the saturation of signal intensities. Therefore, the expression ratio of the α-actin isoform mRNAs were investigated using quantitative RT-PCR. Quantitative RT-PCR was carried out with one primer set, which amplified different length products from the two isoforms about both 480 bp for actin 1 and actin 2b mRNAs, and about 680 bp for actin 2a mRNA showing to the different lengths of their 3' non-coding regions (Fig. 14 B). Direct sequencing confirmed that the RT-PCR products were those of the expected actin isoforms. The results showed that there was differential expression of the two isoforms in each species. The expression ratio of actin 2b to actin 2a mRNA was 4.3 ± 0.18 for *C. yaquinae* and 4.1 ± 0.083 for *C. armatus*, whereas that of actin 1

Table 4. Comparison of the deduced amino acid sequences of actin isoforms from *Coryphaenoides* species and various species

Actin type	Position												
	2	3	54	67	88	137	155	165	228	278	299	354	358
Actin 1	D	E	V	L	T	Q	A	V	A	A	L	S	S
Actin 2a	.	.	.	.	.	.	S	.	.	.	.	.	.
*yaq*actin 2b	.	.	.	P	.	K	S	.	.	.	.	.	.
*arm*actin 2b	.	.	A	.	.	K	S	.	.	.	.	.	.
Pufferfish 1	.	D	.	.	.	.	.	.	.	.	.	.	.
Pufferfish 2	.	D	.	.	S	.	S	I	G	T	.	.	.
Walleye pollack 1	.	.	.	.	.	.	.	.	.	.	.	.	.
Walleye pollack 2	.	.	.	.	.	.	S	.	.	.	.	.	.
Atlantic salmon	.	D	.	.	.	.	S	I	.	.	.	A	T
Carp	.	D	.	.	.	.	.	.	.	.	.	.	T
Zebrafish	.	.	.	.	.	.	.	.	.	.	.	.	.
Medaka	.	.	.	.	.	.	.	.	.	.	.	.	.
Tilapia	.	D	.	.	.	.	.	.	.	.	.	.	.
Chicken	E	D	.	.	.	.	S	I	.	T	M	.	T

"A dot indicates the amino acid residue identical to that in actin 1. Numbers above the sequence indicate the residue position. *yaq*actin 2b and *arm*actin 2b are those cloned from *C. yaquinae* and *C. armatus*, respectively. The GenBank accession numbers for species lists are described in Materials and Methods in Section 2 in Chapter 2. "

to actin 2a was 0.67 ± 0.034 for *C. acrolepis*. The author examined further the ratio at the protein level using two-dimensional electrophoresis. The abundance ratio of actin 2b to actin 2a was 4.8 ± 0.087 for *C. yaquinae* (Fig. 15) and 4.5 ± 0.18 for *C. armatus* (Fig. 15), respectively. The isoforms from *C. acrolepis*, which have the same isoelectric point (5.23) estimated, could not be separated by this electrophoresis method (Fig. 15). These results in the electrophoresis were not affected by dephosphorylation using *Escherichia coli* alkaline phosphatase.

Discussion

One of the most important characteristics of α-actin from deep-sea fish, which enable them to inhabit deep-sea environments, was the extremely small δV associated with actin polymerization, resulting in a lower critical concentration of actin in polymerization at high pressures. Because the small δV was observed even at atmospheric pressure, it is due to actin-actin interactions and not to the relative compressibilities of G- and F-actin. On the basis of the X-ray crystallography structure of actin, however, there are no amino acid substitutions in the regions of actin-actin contact between actin molecules from abyssal and other species.

Two abyssal *Coryphaenoides* species, *C. armatus* and *C. yaquinae*, have three unique amino acid substitutions, V54A or L67P, Q137K and A155S, in comparison with α-actin from non-abyssal *Coryphaenoides* species. The functional significance of these three substitutions in the adaptation to high pressure will be described in Chapter 3.

This study showed that each of non-abyssal species, *C. acrolepis* and *C. cinereus*, have two α-actin isoforms, and that the two isoforms in each species differed by a single amino acid substitution at the 155th residue, Ala-155 in α-actin 1 and Ser-155 in α-actin 2a. The amino acid sequences of α-actin 1 and α-actin 2 from pufferfish (Venkatesh *et al*., 1996) and walleye pollack have the same substitution of A155S (Table 4). carp, goldfish, tilapia and medaka have a single actin isoform with Ala-155, which has not been found in species except fish. These findings suggest that α-actin 1 with Ala-155 is the common isoform of α-actin in fish. On the other hand, the muscles from rabbit (Collins and Elzinga, 1975), chicken (Chang *et al*., 1984), mouse (Hu *et al*., 1986), human (Hanauer *et al*., 1983) and frog (Stutz and Spohr, 1986) contain only one α-actin with Ser-155. The Ala-155 actin variant is not disadvantageous for freshwater fish such as carp, because they do not experience high pressures as

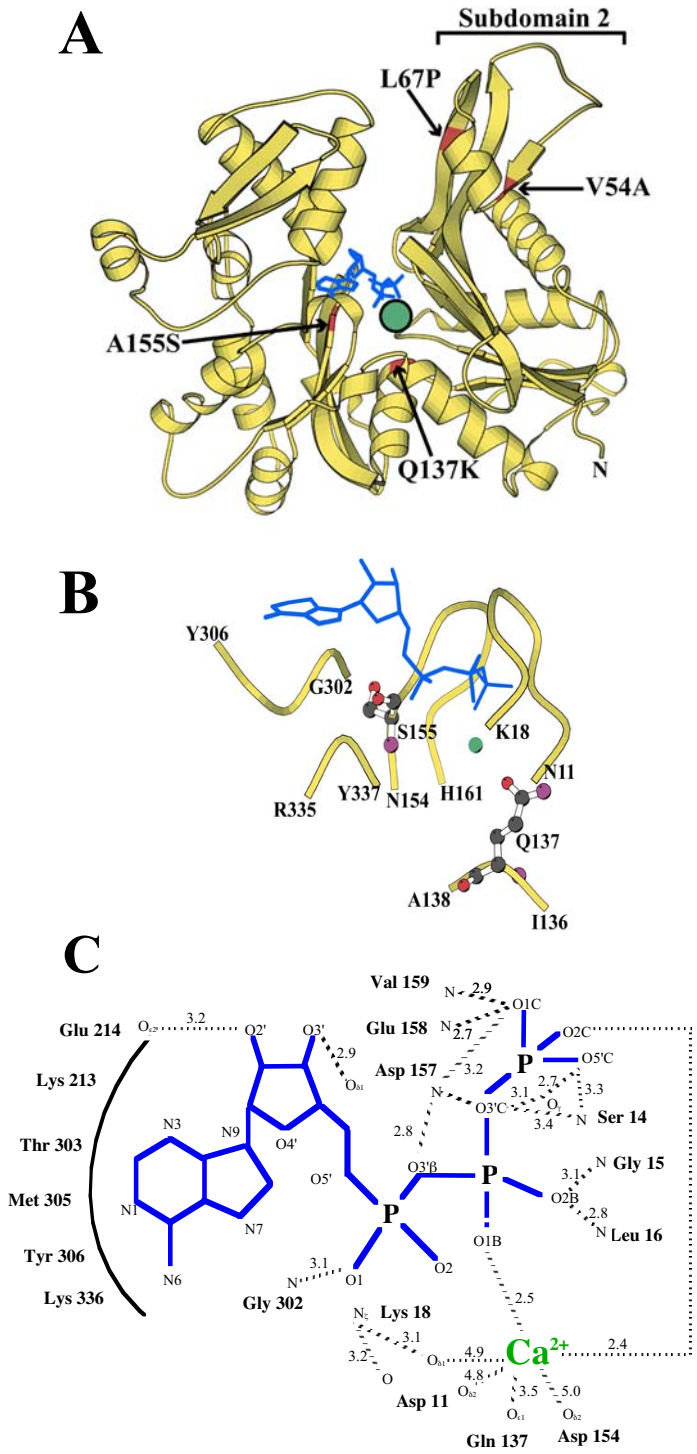

Fig. 13. The structure of the actin bound to ATP and Ca^{2+}. Blue sticks and a greenball represent ATP and Ca^{2+}, respectively. (A) Ribbon drawing of actin (Kabsch *et al*., 1990). The substitutions found in this study are colored red. (B) Environment of ATP and Ca^{2+} bound to the actin. Gln-137 and Ser-155 are represented by ball and stick. Atoms C, O and N are colored grey, red and purple, respectively. Images A and B are created using MOLSCRIPT (Kraulis, 1991). (C) Schematic drawing for interactions of ATP with actin and Ca^{2+} (Kabsch *et al*., 1990). Distances between atoms are given in Å. Amino acids of actin are specified near the circular segment in the left part of the drawing from a pocket covering the adenine.

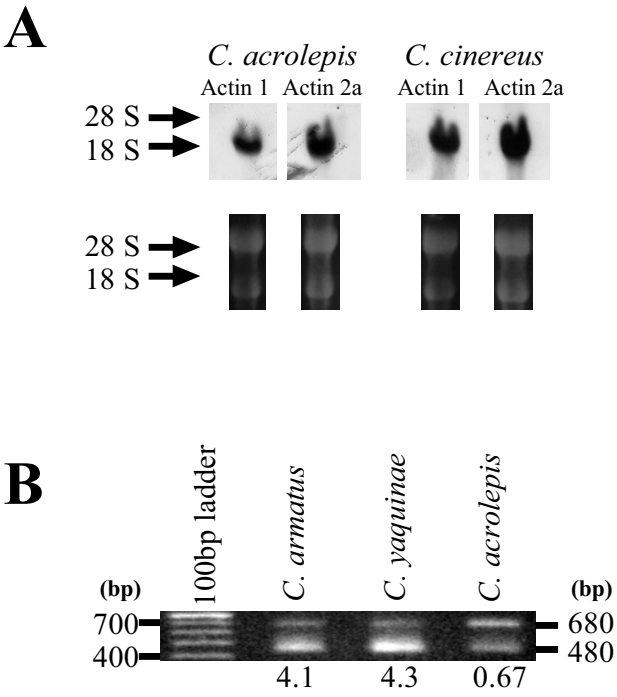

Fig. 14. Northern blot analysis and quantification of actin isoform mRNAs. (A) Northern blot analysis of *C. acrolepis* and *C. cinereus* α-skeletal actin isoform mRNA. The ethidium bromide-stained gels show 28S and 18S rRNA (lower panel), and the separated RNA bands were blotted onto nylon membranes and hybridized with the specific probe for α-skeletal actin 1 and α-skeletal actin 2a (upper panel). (B) RT-PCR analysis for the actin genes from *C. armatus*, *C. yaquinae* and *C. acrolepis*. Numbers indicate the ratios of actin 2b to actin 2a for *C. armatus* and *C. yaquinae*, and actin 1 to actin 2a for *C. acrolepis*.

described in Chapter 3. The molecular phylogenetic tree based on the coding region sequences of α-actin cDNAs clearly showed that actin 2a diverged from actin 1 (Fig. 12). α-actins cloned from freshwater fish so far are the Ala-155 variant. This suggests that the Ala-155 actin is necessary for living in freshwater conditions, and it in marine fish may be a remnant from an ancestral fish. Deep-sea fish, which live under an environment where actin 1 does not completely function as described in Chapter 3, probably have the actin 1 gene but do not express it. The author cannot, however, understand the functional significance of alanine residue at position 155 in actin.

Actin consists of two domains which are historically called large and small domains, although they are nearly in the same size. The small domain contains subdomains 1 [amino acid (a.a.) residues 1-32, 70-144 and 338-372] and 2 (a.a. residues 33-69), while the large domain contains subdomains 3 (a.a. residues 145-180 and 270-337) and 4 (a.a. residues 181-269) (Kabsch *et al*., 1990) (Fig. 16).

The amino acid at the 155th residue with the Ala to Ser substitution in the two isoforms of α-actin found in the present study is located in subdomain 3. Actin binds ATP by sandwiching β- and γ-phosphates of ATP between two structurally equivalent β-hairpins (residues 11-18 and 154-161), which belong to homologous subdomains 1 and 3. ATP forms a bridge between the small and large domains, and thereby prevents the unfolding of the protein and stabilizes the G-actin structure

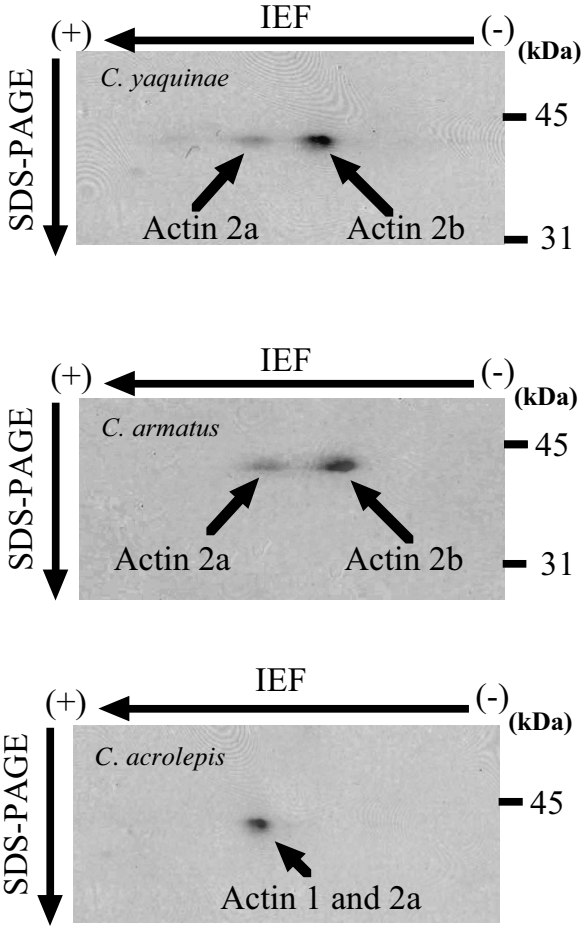

Fig. 15. Quantification of actin isoforms. Two-dimensional electrophoretic patterns of actin from *C. yaquinae*, *C. armatus* and *C. acrolepis*

(Kabsch *et al*., 1990). ATP is closely associated with a divalent cation in actin, Mg^{2+} in native actin and Ca^{2+} in actin-DNaseI crystals, and regulates the polymerization of actin following hydrolysis of ATP (Carlier, 1991; Kinosian *et al*., 1991; Reisler, 1993). Ser-14 of the hydroxyl group is one of six residues (Ser-14, Gly-15, Leu-16, Asp-157, Gly-158 and Val-159) involved in stabilizing the position of the phosphates of ATP, and near to the 155th residue in configuration (Kabsch *et al*., 1990) (Fig. 13 C). The Ser to Ala substitution at the 14th residue results in a 40- to 60-fold decrease in the affinity of actin for ATP and a rapid polymerization of actin than wild type (Chen *et al*., 1995). Ser to Ala substitution at the 155th found in the present study also might influence the affinity of actin for ATP and/or the divalent cation. Swezey and Somero (1982) reported that the Ser-155 actin from *C. armatus* had a high heat stability. Torigai and Konno (1997) also reported that the heat denaturation of α-actin from carp, rainbow trout and walleye pollack is affected by the concentration of ATP, and that their ATP-bound forms are as stable as rabbit α-actin. In *Saccharomyces cerevisiae*, the Ser to Ala substitution at the 14th leads to a temperature-sensitive phenotype in vivo, and temperature-sensitive polymerization in vitro (Chen and Rubenstein, 1995). These findings suggest that the affinity of actin to ATP influences the heat stability of actin and that α-actin with Ala-155, which is thought to be the common isoform in fish, has weaker affinity for ATP than α-actin with Ser-155 of birds, mammals and amphibians. This is supported by the fact that carp G-actin with Ala-155 and *C. acrolepis* actin with the mixture of Ala-155 and Ser-155 isoforms polymerize more rapidly than chicken and abyssal actins with

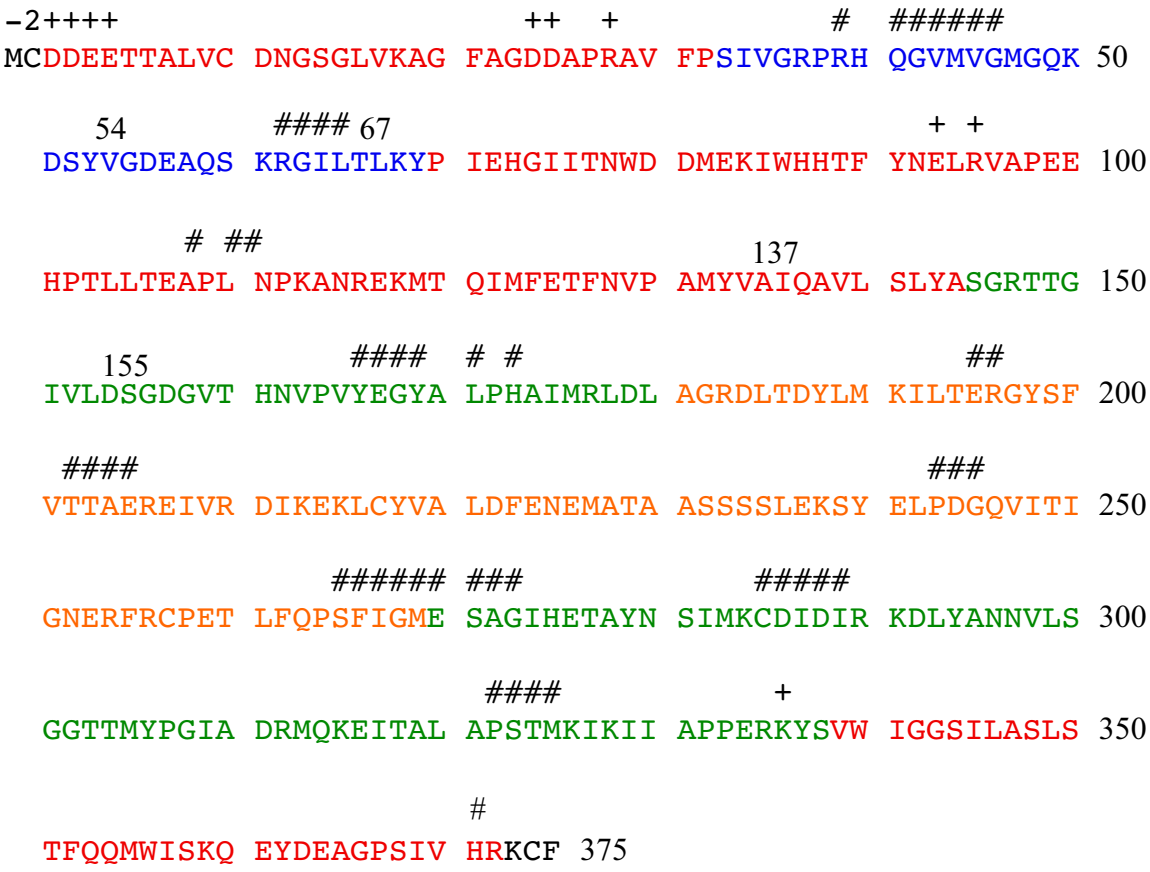

Fig. 16. Amino acid sequences of *Coryphaenoides* actin 1. Symbols # and + represent the binding sites to other actin monomers and myosin, respectively. Amino acid residues constructing subdomains 1, 2, 3 and 4 are indicated by red, blue, green and orange characters, respectively.

Ser-155 only (Fig. 10 A).

Chapter 3. Identification of amino acid residues enabling α-actin from the deep-sea fish to adapt to high pressures

In Chapter 2, the author cloned the α-actin cDNAs from two abyssal *Coryphaenoides* species, *C. armatus* and *C. yaquinae*, and identified three unique amino acid substitutions, V54A or L67P, Q137K and A155S, by comparison with α-actin from non-abyssal *Coryphaenoides* species. The X-ray crystallography structure of actin shows that residues 54 and 67 are located in different β-sheets of subdomain 2, and that residues 137 and 155 are located in the Ca^{2+}- and ATP-binding sites, respectively (Kabsch *et al.*, 1990) (Fig. 13).

Actin contains a tightly bound divalent cation (Ca^{2+} or Mg^{2+})in a deep hydrophilic pocket formed by the β- and γ-phosphates of the bound ATP and actin residues Asp-11, Gln-137, and Asp-154 (Kabsch *et al.*, 1990; Estes *et al.*, 1992) (Fig. 13). Although actin has a higher affinity for Ca^{2+} than for Mg^{2+}, much higher cellular concentration of Mg^{2+} indicates that Mg^{2+} will be the main cation bound in vivo (Estes *et al.*, 1992). Actin also binds ATP by sandwiching the β- and γ-phosphates of ATP of between two structurally equivalent β-hairpins consisting of residues 11-18 and 154-161, which locate in homologous subdomains 1 and 3 (Kabsch *et al.*, 1990; Otterbein *et al.*, 2001).

In this chapter, the author investigated the effect of Q137K and A155S substitutions on the binding of actin with divalent cation and nucleotide at various pressures. In addition, he investigated the effect of pressures on the actin structure by measuring the intrinsic tryptophan fluorescence spectrum of G-actin and the DNaseI inhibitation.

Materials and Methods

Material

Samples used were the same in Chapter 2.

Actin

Actin was prepared as described in Chapter 2.

Quin 2 assay

The dissociation rate constant of Ca^{2+} from actin was determined by measuring the increase of fluorescence of 8-amino-2-[(2-amino-5-methylphenoxy) methyl]-6-methoxyquinoline-N,N,N',N'-tetraacetic acid (Quin 2) (Kinosian *et al.*, 1993). Ca^{2+}-actin of 5 μM dialyzed against G-buffer free of $CaCl_2$ was added to 100 μM Quin 2 and 0.2 mM $MgCl_2$ and the changes in fluorescence are measured at excitation and emission wavelengths of 340 and 500 nm, respectively, at 4℃ in a high-pressure cell. The reaction rates were determined by the following relevant thermodynamic equation:

$(\delta \ln k/\delta P) = -\delta V\#/RT$,

where k is rate constant for the reaction, P is pressure, the $V^{\#}$ is activation volume, R is gas constant and T is absolute temperature (Morild, 1981). The apparent binding rate constant and apparent volume change ($\delta V^{\#}$) of the Ca^{2+} dissociation reaction were determined as described previously (Waechter and Engel, 1975; Morild, 1981).

Nucleotide exchange assay

The apparent binding rate constant of $1,N_6$-etheno-ATP (δ-ATP) with actin was determined by displacing bound ATP with a large molar excess of δ-ATP such that the back reaction of ATP binding to actin became negligible (Kinosian *et al.*, 1993). Actin of 9 μM dialyzed against G-buffer free of ATP was converted into Mg^{2+}-G-actin and added to 0.1 mM ε-ATP, and the changes in fluorescence was measured at excitation and emission wavelengths of 340 and 410 nm, respectively, at 4℃ in a high-pressure cell. The apparent binding constant and apparent $\delta V^{\#}$ in the ATP dissociation reaction were determined as described previously (Morild, 1981; Waechter and Engel, 1975).

Intrinsic tryptophan fluorescence

The intrinsic tryptophan fluorescence spectrum of Ca^{2+}-G-actin at 6.25 μM was recorded using a high-pressure cell at excitation and emission wavelengths of 290 and 320-360nm, respectively, at 4℃ (Lehrer and Kerwar, 1972).

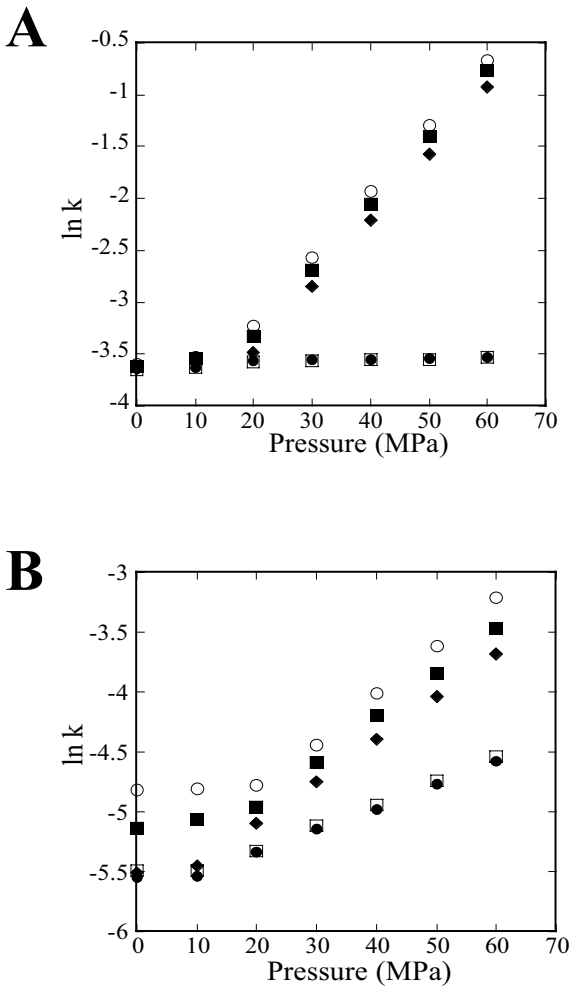

Fig. 17. Logarithmic plots of the dissociation rate constant of Ca^{2+} (A) and ATP (B) in actin at various pressures. Symbols are as follows: abyssal *C. yaquinae* (●); abyssal *C. armatus* (□); chicken (◆); carp (○); non-abyssal *C. acrolepis* (■). Values are means ± S.D. of four independent experiments. Errors for all experiments are too small to show error bars.

DNaseI inhibition assay

The DNaseI inhibition assay was performed as described previously (Blikstad *et al.*, 1978). In brief, DNaseI, either alone or combined with G-actin at various actin/DNaseI ratios, was added to a control or salmon sperm DNA solution containing 100 mM Tris-HCl (pH 7.6), 4 mM $MgSO_4$ and 1.8mM $CaCl_2$ and the change in absorbance at 260 nm (A_{260}) was recorded continuously at 20℃ in a high-pressure cell. DNaseI activity was calculated from the linear part of the plot of the increase in A_{260} versus time.

Results

Sequence analysis showed that actin 2b from abyssal species has lysine at position 137. The other isoform from abyssal species and all actins from non-abyssal species have glutamine at this position, and all actin isoforms from abyssal species have serine at residue 155. Actin binds ATP by sandwiching the β- and γ-phosphates of ATP between two structurally equivalent β-hairpins (residues 11-18 and 154-161), which locate at homologous subdomains 1 and 3 (Kabsch *et al.*, 1990; Otterbein *et al.*, 2001). Actin also contains a tightly bound divalent cation (Ca^{2+} or Mg^{2+}) in a deep hydrophilic pocket formed by the β- and γ- phosphates of the bound ATP and actin residues Asp-11, Gln-137, and Asp-154 (Kabsch *et al.*, 1990; Estes *et al.*, 1992). Although actin has a higher affinity for Ca^{2+} than for Mg^{2+}, much higher cellular concentrations of Mg^{2+} indicate that Mg^{2+} is the main cation bound in vivo (Estes *et al.*, 1992). The author investigated the effect of high pressures on the divalent cation dissociation and nucleotide binding of actin at various pressures (Fig. 17). Surprisingly, both dissociation rate constants of actin from abyssal species were much less affected by high pressures than those of the other actins, which increased rapidly at pressures higher than 20 MPa. The dissociation rate constant of Ca^{2+} in abyssal actins did not differ markedly from those of the other actins at atmospheric pressure, indicating that actins bind to Ca^{2+} with almost the same strength. The differences in the dissociation rate constant of ATP suggests that Ser-155 actin binds ATP more tightly than Ala-155 actin, as pointed out in Chapter 2. The apparent $\delta V^{\#}$ in the Ca^{2+} dissociation reaction at pressures greater than 20 MPa was estimated as -4.27 ± 1.86cm^3 mol^{-1} for abyssal species actins and -154.3 ± 0.583cm^3 mol^{-1} for other actins. The apparent δV# in the ATP dissociation reaction at more than 20 MPa was also determined to be -43.4 ± 0.47cm^3 mol^{-1} or more for abyssal actins, and to be -80.4 ± 1.44, -90.0 ± 1.59 and -84.2 ± 0.995cm^3 mol^{-1} for chicken, carp and non-abyssal actin, respectively. These results indicate that the smaller effect on both

dissociation rate constants of pressure for abyssal actins results from Q137K and the differences in both constants among carp, chicken and non-abyssal species do from A155S.

Effects of pressures on intrinsic tryptophan fluorescence spectrum

Actin contains four tryptophan residues at positions 79, 86, 340 and 356 (Fig. 18), which are all located in subdomain 1. The emission maximum of fluorescing tryptophan residues is 350 nm in a neutral water solution, but shifts to shorter wavelengths in a hydrophobic environment such as in the interior of a folded protein (Kuznetsova *et al*., 1999). To investigate the effect of pressures on the actin structure, the author measured the intrinsic tryptophan fluorescence of Ca^{2+}-G-actin at various pressures. Although the emission maximum did not shift, the fluorescence intensity of actins from chicken, carp and non-abyssal species began to decrease at only 10 MPa and in particular the decrease for carp actin was larger than that for chicken and non-abyssal actins. By contrast, the fluorescence of abyssal actins did not change even at 60 MPa (Fig. 19). Actin affinity for Ca^{2+} is greater than for Mg^{2+} (Estes *et al*., 1992), but there were no differences in the fluorescence intensity between the Ca^{2+}- and Mg^{2+}-G-actin forms for all actin species. These results indicate that high pressures change the environment of tryptophan residues in actin, in other words, the structure of actin subdomain 1 including one of β-hairpins that sandwiches the β- and γ-phosphates of ATP and Ca^{2+}-binding sites.

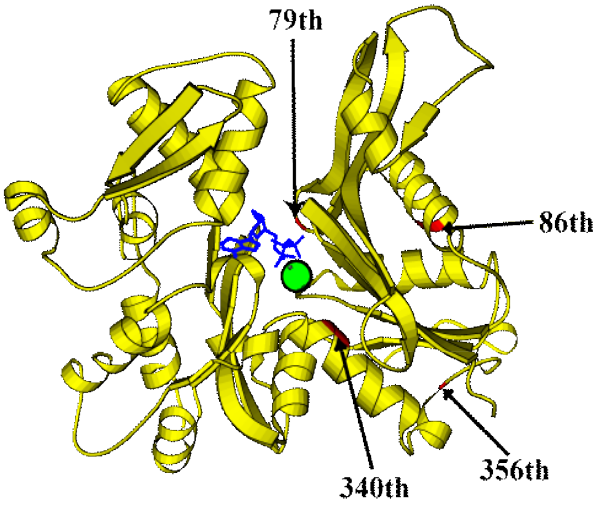

Fig. 18. The positions of tryptophan residues in G-actin. Tryptophan residues at 79, 86, 340, and 356 are colored red. Blue bars indicate ATP, and a green ball does Ca^{2+}.

DNaseI inhibition assay

Actin 2b of the two abyssal species contains either a V54A or an L67P substitution. The X-ray crystallography structure shows that these residues are located in subdomain 2 (Fig. 13 A, 16). In actin-DNaseI interactions, DNaseI primarily contacts the DNaseI-binding loop of residues 40-48 in subdomain 2 and interacts slightly with Thr-203 and Glu-207 in subdomain 4 (residues 181-269) (Kabsch *et al*., 1990; Khaitlina *et al*., 1993). To investigate whether these substitutions, V54A and L67P, affect the actin-DNaseI interaction, the author performed a DNaseI inhibition assay at various pressures (Fig. 20). Notably, the author found that abyssal actins inhibited DNaseI activity even at 60MPa, whereas other actins showed little activity at 60MPa. In addition, carp actin was almost inactive at 30MPa. The decrease in DNaseI inhibition activity of actin from 0.1MPa to 60MPa was higher for *C. armatus* actin (23.9%) than for *C. yaquinae* actin (17.2%), in agreement with the fact that *C. yaquinae* lives at higher depths (Endo and Okamura, 1992). These decreases would depend on the denatured state of actin 2a by high pressures, which slightly exists in the muscles of both *C. yaquinae* and *C. armatus* as shown by two-dimensional electrophoresis (Fig. 15). These finding clearly indicate actins from abyssal species were bound to DNaseI even at 60 MPa, suggesting that the substitutions in subdomain 2 of actins from abyssal species are most likely to reduce the increased volumes that occur with the interaction of actin to DNaseI. However, the mechanism underlying the reduction in volume remains unclear.

Discussion

Subdomain 2 consisting of residues 33-69 is probably the most flexible part among all actin

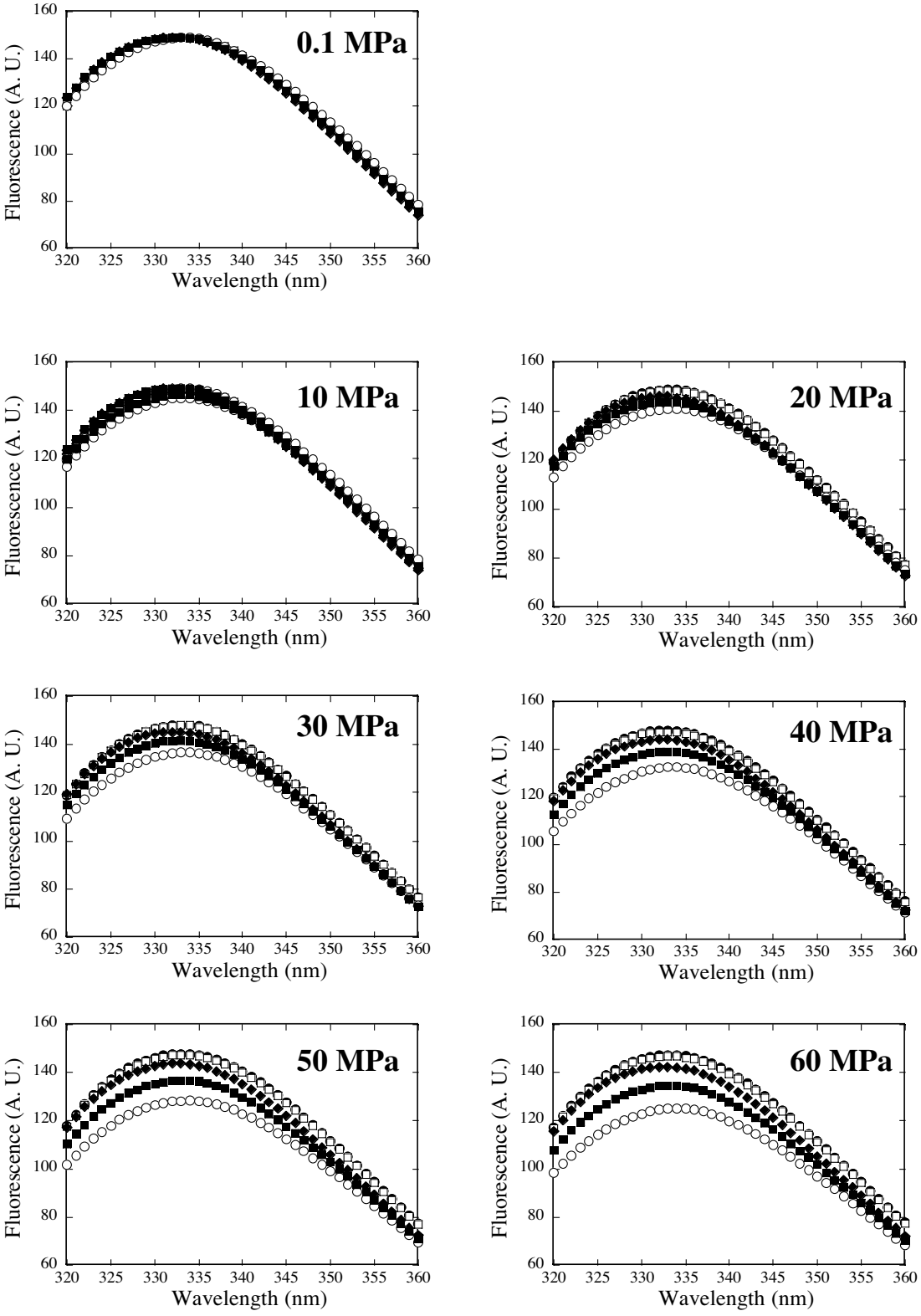

Fig. 19. Effects of pressures from 0.1 MPa to 60 MPa on the intrinsic tryptophan fluorescence spectrum of Ca^{2+}-G-actins. Symbols are as follows: abyssal *C. yaquinae* (●); abyssal *C. armatus* (□); chicken (◆); carp (○); non-abyssal *C. acrolepis* (■).

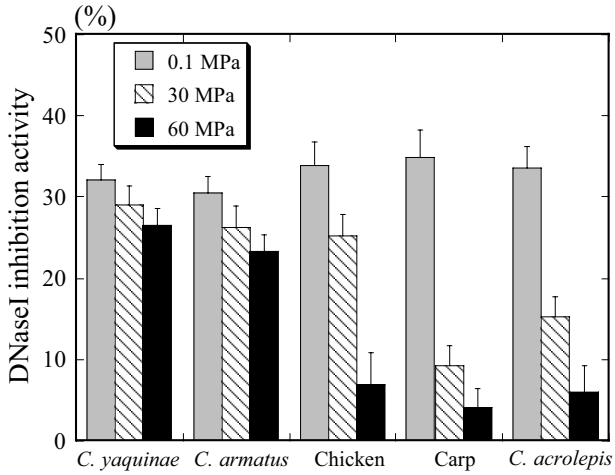

Fig. 20. Effects of pressures on the DNaseI inhibition activity of G-actin. The experiments for each actin were performed at the same ratios of actin/DNaseI at every pressure. Values are means ± S.D. of four independent experiments.

subdomains (Moraczewska *et al*., 1999) and its conformational change after ATP hydrolysis and Pi release from ADP-Pi during actin polymerization plays a critical role in filament dynamics (Orlova and Egelman, 1993; Belmont *et al*., 1999). Residues 41-45 in subdomain 2 of one actin molecule interact with two other actin molecules, one at residues 166-169 and the other around residue 375 (Holmes *et al*., 1990). The author's results show that the DNaseI-binding loop consisting of residues 40-48 in subdomain 2 of abyssal actin can interact with DNaseI even at high pressures, therefore, the author proposes that the abyssal actin also undergoes actin-actin interactions at high pressures without a large increase in volume. Clearly, such a possibility is provided by the V54A or L67P substitution in the β-sheet of subdomain 2 of actin from the abyssal species. Such substitution is known to weaken the formation of β-sheet structure (Levitt, 1978). However, the mechanism of these substitutions reducing δV associated with actin polymerization has not been understood yet.

The dissociation of ATP and Ca^{2+} in particular from actins of abyssal species was less affected by pressure than that of actins from other species. This tolerance to high pressures of actins from abyssal species is due especially to the A155S and Q137K substitutions. These substituted amino acids, which increase the negative value of $\delta V^{\#}$ in both the Ca^{2+} and the ATP dissociation reactions, prevent these reactions from being strongly accelerated by high pressures. Ca^{2+} lies below the bound ATP on the pseudo-twofold axis and is coordinated by four water molecules, which are held in place through interactions with the side chains of Asp-11, Gln-137 and Asp-154, and two O atoms from the β- and γ-phosphates of the bound ATP (Kabsch *et al*., 1990; Otterbein *et al*., 2001). Actin, Hsp70 molecular chaperones, hexokinase, and sugar kinases share structural homology and can therefore be considered members of a superfamily (Flaherty *et al*., 1991; Bork *et al*., 1992; Hurley, 1996) (Fig. 21). In this superfamily, the amino acid in the equivalent position to Gln-137 found in most actin is usually Asp or Glu, i.e., a negatively charged residue. Surprisingly, actins from abyssal species have positively charged residue Lys at position 137. The Q137K substitution thus changes the coordination of Ca^{2+} with protein. The repulsion between Ca^{2+} and Lys-137 would prevent the Ca^{2+} from being pushed into the bottom of the interdomain cleft by high pressures. On the other hand, another study showed that the rate of ligand entry from solvent to protein interior is increased if the space available to the ligand in the protein pocket is increased by substituting an amino acid residue with a smaller side chain (Gibson *et al*., 1992). The substitution of amino acids with larger side chains (Gekko and Hasegawa, 1986) such as Q137K and A155S might prevent both Ca^{2+} and ATP dissociation reactions from being accelerated by high pressures. Previous studies have reported that high pressures can slightly affect an apparent Km for substrate in some dehydrogenases from deep-sea fish (Somero, 1990; Siebenaller, 1991; Somero, 1992; Siebenaller and Murray, 1995; Gibbs, 1997). The author's results suggest a mechanism for stabilizing enzyme-substrate interactions under elevated pressures.

The Q137K and A155S substitutions allows that actins other than those of abyssal species to have larger cavities in the protein pocket, owing to the presence of amino acid residues with smaller side chains. The results from Quin 2 and nucleotide exchange assays showed, however, that these

cavities remained unchanged at high pressures. On the other hand, the author's observations here (Fig. 17) and previous studies (Swezey and Somero, 1982) demonstarated that abyssal species actins formed tighter associations with ATP and Ca than other species actins did. The structure of actin is maintained not only by its endogenous weak non-covalent bonds but also by bound ATP and Ca (Kinosian *et al*., 1993). Thus, tightly bound ATP and Ca^{2+} in abyssal species actins presumably maintain the actin structure from inside, like a pillar. In support of this, carp G-actin was much softer (Fig. 19) and δV of carp actin increased with high pressures (Fig. 10 C), which indicated that for carp actin the coefficient of compressibility for G-actin state was larger than that for F-actin state. Thus, the occurrence of Ala-155 would explain why carp actin was able to polymerize only at 20 MPa or less.

The Ala-155 variant of actin would not be disadvantageous for freshwater fish such as carp, because they do not experience high pressures. However, it would be the disadvantageous for many marine fish. The molecular phylogenetic tree for actin genes clearly showed that actin 2a diverged from actin 1 (Fig. 12). All α-actins cloned from freshwater fish so far are Ala-155 variant, suggesting that Ala-155 actin is necessary for living in freshwater conditions and may be a remnant from an ancester for marine fish. Deep-sea fish, which live in an environment where actin 1 does not function, probably have the actin 1 gene but do not express it. In the superfamily including actin,

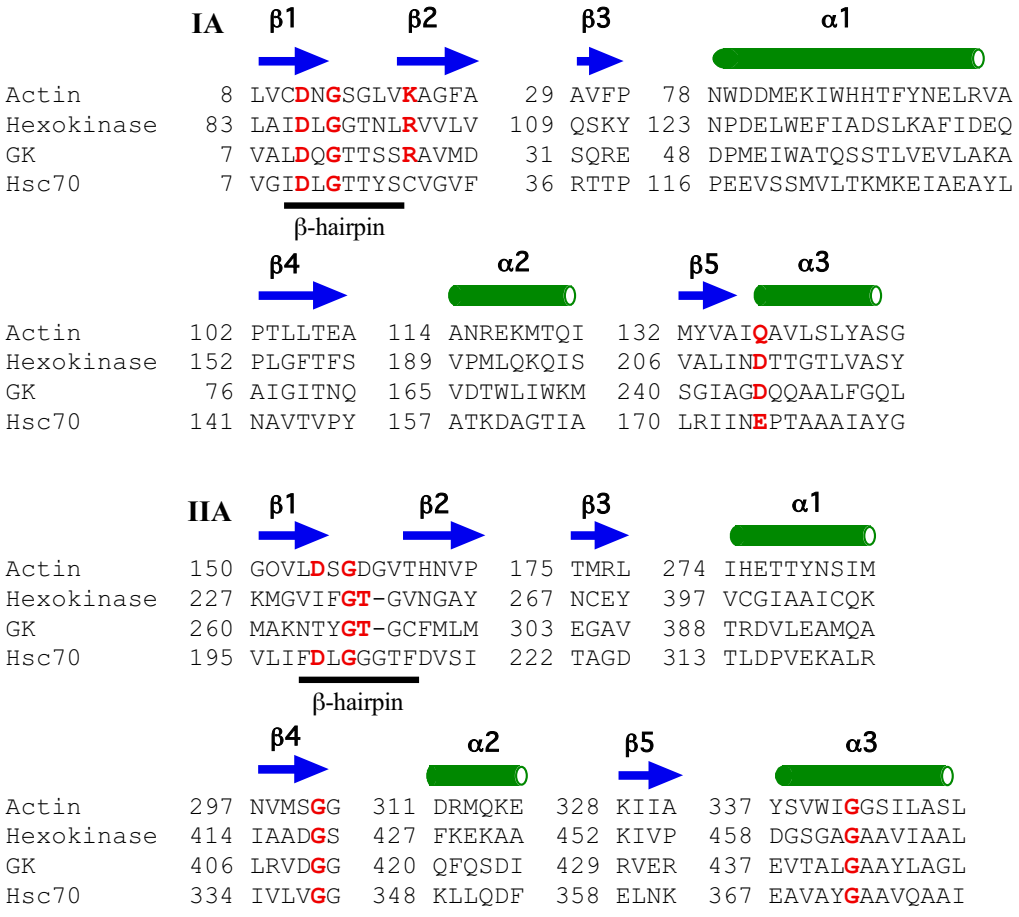

Fig. 21. Sequences of structurally conserved regions of actin related superfamily members (Hurley, 1996). The hexokinase sequence is numbered according to the translated gene sequence of yeast hexokinase B. The alignment is based solely on three-dimensional structural equivalence. Only regions that are equivalent structurally in all four proteins are shown. Red residues are either identical in all sequences or have structurally equivalent roles in the nucleotide binding site.

Hsp70 molecular chaperones, hexokinase, and sugar kinases, the amino acid in the position equivalent to 155 in common actin is usually uncharged polar or non-polar amino acid (Flaherty *et al*., 1991; Bork *et al*., 1992; Hurley, 1996). Therefore, Ala-155 actin found in many fish would be accepted without any problem in their polarity. However, the author does not understand the full significance of Ala-155 in actin from freshwater fish.

A large portion of the present data was collected from experiments using a mixture of actin isoforms prepared from intact muscle, where the author was unable to separate from each other by biochemical techniques. Although the expression of recombinant proteins in microorganisms is a powerful tool, the system for actin, in particular α-actin, has not been established yet because of various problems, such as the formation of inclusion bodies and low yield of expressed proteins (Buzan *et al*., 1990). The author is trying to set up expression system of abyssal species actin for future studies.

Chapter 4. General discussion

How do deep-sea fish adapt to high pressures of approximately 60MPa? Many marine biologists have been interested in and made the efforts to unravel this question. Adaptations of Organisms to the environment can be categorized as "tolerance adaptations" which enable an organism to survive in a given environment, and "capacity adaptations" involving regulation of rates of physiological processes, *e.g.* quantitative or qualitative changes of gene expression (Yamashita *et al*., 1996; Hirayama and Watabe, 1997; Battersby and Moyes, 1998; Hardewig *et al*., 1999; Kikuchi *et al*., 1999; Watabe, 2002; Imamura *et al*., 2003; Itoi *et al*., 2003). Previous advanced studies have shown that proteins from deep-sea fish functioned even under high hydraulic pressures (Somero, 1990, 1992; Siebenaller, 1991; Siebenaller and Murray, 1995; Gibbs, 1997; Yancey *et al*., 2001). Therefore, it seems that deep-sea fish selected the tolerance adaptations. While amino acid sequences of proteins, which adapt to high hydraulic pressures, had not been determined, the results in this study indicated, for the first time, amino acid sequences of the unique proteins (Table 4).

Which amino acid substitution contributes to high pressure adaptation of proteins from deep-sea fish, and how does it do? If a reaction proceeds with an increase of volume, increased pressures will inhibit the reaction. The processes, which result in a decrease of volume, will be enhanced by an increase of pressures. Although only reaction with no volume change does not depend on pressures, most biological processes are followed by volume changes. Therefore, proteins from deep-sea fish would change the reaction volume so that their biological reaction is not influenced by high pressures. Thus, two assumptions about the site of amino acid substitution, possibly occurring in proteins from deep-sea fish, were advocated (Somero, 1992). First, substitutions inside proteins and at the protein-water interface bring about alterations in the packing efficiency and hydration volume change, respectively. The latter is that the reaction volume change is produced by amino acid substitution at the ligand binding or subunit contact sites. The present results from deep-sea fish α-actin supported the latter assumption. The first assumption will be supported in other proteins from deep-sea fish, *e.g.* LDH, in the near future.

The present results also showed the differences in mRNA and protein expressions between actin 2a and actin 2b, and between actin 1 and actin 2a (Fig. 14 B, 15). These differences indicate that an actin isoform suitable for adaptation to the environment, in which each fish live, is more abundant at both mRNA and protein levels. These results suggest the capacity adaptations in deep-sea fish by the quantitative change of gene expression.

Recent studies showed that a high concentrating TMAO (trimethlamine oxide) in muscle of deep-sea fish offsets an inhibitory effect of high hydrostatic pressures on biological processes (Yancey and Siebenaller, 1999; Yancey *et al*., 2001; Yancey *et al*., 2002). Other studies also indicated that high pressures have the membrane-ordering effects on deep-sea organisms, which change membrane lipid composition and fluidity, by increasing the proportion of unsaturated fatty acids (Phleger and Laub, 1975; Cossins and Macdonald, 1984, 1986, 1989). The membrane lipids alone are not, however, related

to high pressure adaptation in the case of sodium pump (Gibbs and Somero, 1990; Gibbs, 1995). As the present results and those studies showed, deep-sea fish advanced into the largest space among the ocean (Fig. 1) not only by amino acid substitution in proteins but also by the changes in concentration of low molecules such as TMAO and membrane composition. Thus, the advancement into deep-sea would be result of integration of many contingencies which happened according to the neutral theory of molecular evolution (Kimura, 1968). The separation between abyssal and non-abyssal *Coryphaenoides* is estimated to have occured approximately 3.2-7.6 million years ago (Fig. 8).

There are a number of actin-binding proteins (Pollard and Cooper, 1986) and, as a result, actin is one of the most conserved proteins with only limited variations. The present results have shown that a novel function of a protein that enables species to adapt to a new environment can be achieved by a very few amino acid substitutions in key functional positions. Moreover, the molecular phylogenetic tree based on α-actin coding region showed that actin 2a diverged from actin 1 and actin 2b did from actin 2a, again by a very few amino acid substitutions. Freshwater fish had only Ala-155 actin, i.e., actin 1 (Table 4, Fig. 12). Thus, when teleosts advanced from freshwater to sea and continuously did from surface to abyssal zone, they would have duplicated α-actin gene each time. Such observations are consistent with the Perutz's theory of protein speciation (Perutz, 1983), the theory of gene duplication (Ohno, 1970) and the prediction from the molecular phylogenetic tree based on mitochondrial DNA analysis (Fig. 6, 7, 8).

Low temperature (1-4℃) is also one of physical factors characteristic of the deep-sea. A_4-lactate dehydrogenase (A_4-LDH; 1.1.1.27) of Antarctic notothenioid fish has been well known to adapt to extreme cold temperatures (Somero, 1995), and those amino acid sequences were recently determined (Fields and Somero, 1998). A_4-LDH of deep-sea fish has also been known to adapt to high pressures and low temperatures (Somero, 1990; Siebenaller, 1991; Somero, 1992; Siebenaller and Murray, 1995; Gibbs, 1997). High pressures together with low temperatures can slightly affect an apparent Km of substrate in some dehydrogenases from deep-sea fish. It is of interest whether deep-sea fish have unique substitutions similar to those found in A_4-LDH of Antarctic notothenioid fish.

It has been considered that new species occur when gene flow between different populations is interrupted (France and Kocher, 1996). High hydraulic pressures are considered to accelerate the speciation process as a barrier of gene flow even in the absence of absolute geographic isolation, since hydraulic pressures have various effects on biochemical and physiological processes (Siebenaller and Somero, 1978, 1989; Siebenaller, 1991; Childress and Thuesen, 1995; Gibbs, 1995; Siebenaller and Murray, 1995). The present study indicates that differences in the adaptation of α-actin to hydraulic pressures produced the speciation between abyssal and non-abyssal species. Considering that the molecular phylogenetic trees by mtDNA analysis do not clearly define the taxonomic relationships of non-abyssal species, factors other than those participating in biochemical processes, *e.g.* food habit, are most likely to be concerned in the speciation of non-abyssal fish in the upper continental slope.

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References

Anderson S., Bankilar A. T., Barrell B. G., de Bruijn M. H. L., Coulson A. R., Drouin J., Eperon I. C., Nierlich D. P., Roe B. A., Sanger F., Schreier P. H., Smith A. J. H., Staden R., and Young I. G., 1981: Sequence and organization of the human mitochondrial genome. *Nature,* **290**, 457-465.

Belmont L. D., Orlova A., Drubin D. G., and Egelman E. H., 1999: A change in actin conformation associated with filament instability after Pi release. *Proc. Natl. Acad. Sci. USA*, **96**, 29-34.

Blikstad I., Markey F., Carlsson L., Persson T., and Lindberg U., 1978: Selective assay of monomeric and filamentous actin in cell extracts, using inhibition of deoxyribonuclease I. *Cell,* **15**, 935-943.

Bork P., Sander C. and Valencia A., 1992: An ATPase domain common to prokaryotic cell cycle proteins, sugar kinases, actin, and hsp70 heat shock proteins. *Proc. Natl. Acad. Sci. USA,* **89**, 7290-7294.

Battersby B. J. and Moyes C. D., 1998: Influence of acclimation temperature on mitochondrial DNA, RNA, and enzymes in the skeletal muscles. *Am. J. Physiol. Regul. Integr. Comp. Physiol.,* **275**, R905-R912.

Buzan J., Du J., Karpova T., and Frieden C., 1990: Histidine-tagged wild-type yeast actin: its properties and use in an approach for obtaining yeast actin mutants. *Proc. Natl. Acad. Sci. USA,* **96**, 2823-2827.

Caconne A., Milinkovitch M. C., Sborboni V., and Powell J. R., 1994: Molecular biogeography: using the Corsica-Sardinia microplate disjunction to calibrate mitochondrial rDNA evolutionary rates in mountain newts (Euproctus). *J. Evol. Biol.,* **7**, 227-245.

Carlier M. F., 1991: Actin: protein structure and filament dynamics. *J. Biol. Chem.,* **266**, 1-4.

Chang K. S., Zimmer W. E. Jr, Bergsma D. J., Dodgson J. B., and Schwartz R. J., 1984: Isolation and characterization of six different chicken actin genes. *Mol. Cell. Biol.,* **4**, 2498-2508.

Chen X., Cook R. K., and Rubenstein P. A., 1993: Yeast actin with a mutation in the 'hydrophobic plug' between subdomains 3 and 4 (L266D) displays a cold-sensitive polymerization defect. *J. Cell Biol.,* **123**, 1185-1195.

Chen X. and Rubenstein P. A., 1995: A mutation in an ATP-binding loop of *Saccharomyces cerevisiae* actin (S14A) causes a temperature-sensitive phenotype *in vivo and in vitro. J. Biol. Chem.,* **270**, 11406-11414.

Chen X., Peng J., Pedram M., Swenson C. A., and Rubenstein P. A., 1995: The effect of the S14A mutation on the conformation and thermostability of *Saccharomyces cerevisiae* G-actin and its interaction with adenine nucleotides. *J. Biol. Chem.,* **270**, 11415-11423.

Childress J. J. and Thuesen E. V., 1995: Metabolic potentials of deep-sea fishes: a comparative approach, in "*Biochemistry and Molecular Biology of Fishes*" (ed. by Hochachka P. W. and Mommsen T. P.), vol. 5, Elsevier Press, Amsterdam, pp. 175-196.

Cohen J. E., 1994: Marine and continental food webs: three paradoxes? *Philos. Trans. R. Soc. London B*, **343**, 57-69.

Collins J. H. and Elzinga M., 1975: The primary structure of actin from rabbit skeletal muscle. Completion and analysis of the amino acid sequence. *J. Biol. Chem.,* **250**, 5915-5920.

Cossins A. R. and Macdonald A. G., 1984: Homeoviscous theory under pressure: II. the molecular order of membranes from deep-sea fish. Biochim. *Biophys. Acta,* **776**, 144-150.

Cossins A. R. and Macdonald. A. G., 1986: Homeoviscous theory under pressure: III. the fatty acid composition of liver mitochondorial phospholipids of deep-sea fish. *Biochim. Biophys. Acta*, **860**, 325-335.

Cossins A. R. and Macdonald A. G., 1989: The adaptations of biological membranes to temperature and pressure: Fish from the deep and cold. *J. Bioenerg. Biomembranes,* **21**, 115-135.

Dutton P. H., Davis S. K., Guerra T., and Owens D., 1996: Molecular phylogeny for marine turtles based on sequences of the ND4-leucine tRNA and control regions of mitochondrial DNA. *Mol. Phylogenet. Evol.,* **5**, 511-521.

Endo H. and Okamura O., 1992: New records of the abyssal grenadiers *Coryphaenoides armatus* and *C. yaquinae* from the western North Pacific. *Japan. J. Ichthyol.*, **38**, 433-437.

Estes J. E., Selden L. A., Kinosian H. J., and Gershman L. C., 1992: Tightly-bound divalent cation of actin. *J. Muscle Res. Cell Motil.,* **13**, 272-284.

Fabrizio C., Luca B., Lucilla O., Eliana P., Lorenzo C., and Tomaso P., 1996: Molecular phylogeny of grey mullets based on mitochondrial DNA sequence analysis: evidence of a differential rate of evolution at the intrafamily level. *Mol. Phylogenet. Evol.,* **6**, 416-424.

Fajen A. and Breden F., 1992: Mitochondrial DNA sequence variation among natural populations of the Trinidad guppy, *Poecilia reticulata*. *Evolution,* **46**, 1457-1465.

Fields P. A. and Somero, G. N., 1998: Hot spots in cold adaptation: localized increases in conformational flexibility in lactate dehydrogenase A4 orthologs of Antarctic notothenioid fishes. *Proc. Natl. Acad. Sci. USA,* **95**, 11476-11481.

Felsenstein J., 1981: Evolutionary trees from DNA sequences: a maximum likelihood approach. *J. Mol. Evol.,* **17**, 363-376.

Felsenstein J., 1985: Confidence limits on phylogenies: an approach using the bootstrap. *Evolution,* **46**, 1457-1465.

Felsenstein J., 1995: Phylogeny inference package (PHYLIP) Version 3.57c. Department of Genetics, SK-50, University of Washington, Seattle, WA.

Flaherty K. M., McKay D. B., Kabsch W., and Holmes K. C., 1991: Similarity of the three-dimensional structures of actin and the ATPase fragment of a 70-kDa heat shock cognate protein. *Proc. Natl. Acad. Sci. USA,* **88**, 5041-5045.

France S. C. and Kocher T. D., 1996: Geographic and bathymetric patterns of mitochondrial 16 S rRNA sequence divergence among deep-sea amphipods, *Eurythenes gryllus*. *Mar. Biol.*, **126**, 633-643.

Gage J. D. and Tyler P. A., 1991: Deep-sea Biology: A Natural History of Organisms at the Deep-Sea Floor. Cambridge Univ. Press, Cambridge, 504pp.

Garland T. Jr. and Adolph S. C., 1994: Why not to do two-species comparative studies: limitations on inferring adaptation. *Physiol. Zool.,* **67**, 797-828.

Garland T. Jr. and Carter P. A., 1994: Evolutionary physiology. *Annu. Rev. Physiol.,* **56**, 579-621.

Gekko K. and Hasegawa Y., 1986: Compressibilit y-structure relationship of globular proteins. *Biochemistry,* **25**, 6563-6571.

Gibbs A. and Somero G.N., 1990: Pressure adaptation of teleost gill Na^+, K^+-adenosine triphosphatase: role of the lipid and protein moieties. *J. Comp. Physiol. B*, **160**, 431-439.

Gibbs A., 1995: Temperature, pressure and the sodium pump: the role of homeoviscous adaptation, in "*Biochemistry and Molecular Biology of Fishes*" (ed. by Hochachka P. W. and Mommsen T. P.), vol. 5, Elsevier Press, pp. 197-212.

Gibbs A., 1997: Biochemistry at depth, in *Deep-Sea Fishes* (ed. by Randall D. J. and Farrell A. P.), Academic Press, New York, pp. 239-277.

Gutell R. R., Weiser R., Woese C. R., and Noller H.

F., 1985: Comparative anatomy of 16-S-like ribosomal RNA. *Prog. Nucleic Acid Res. Mol. Biol.,* **32**, 155-216.

Gibson Q. H., Regan R., Elber R., Olson J. S., and Carver T. E., 1992: Distal pocket residues affect picosecond ligand recombination in myoglobin. an experimental and molecular dynamics study of position 29 mutants. *J. Biol. Chem.*, **267**, 22022-22034.

Hanauer A., Levin M., Heilig R., Daegelen D., Kahn A., and Mandel J. L., 1983: Isolation and characterization of cDNA clones for human skeletal muscle alpha actin. *Nucleic Acids Res.,* **11**, 3503-3516.

Harasewych M. G., Adamkewicz S. L., Blake J. A., Saudec D., Spriggs T., and Bult C. J., 1997: Phylogeny and relationships of pleurotomariid gastropods (Mollusca: Gastropoda): an assessment based on partial 18 S rRNA and cytochrome c oxidase I sequences. *Mol. Mar. Biol. Biotechnol.,* **6**, 1-20.

Hardewig I., van Dijk P. L. M., Moyes C. D., and Portner, H. O., 1999: Temperature-dependent expression of cytochrome-c oxidase in Antarctic and temperate fish. *Am. J. Physiol. Regul. Integr. Comp. Physiol.,* **277**, R508-R516.

Heremans K., 1982: High pressure effects on proteins and other biomolecules. *Annu. Rev. Biophys. Bioeng.,* **11**, 1-21.

Hirayama Y. and Watabe S., 1997: Structural differences in the crossbridge head of temperature-associated myosin subfragment-1 isoforms from carp fast skeletal muscle. *Eur. J. Biochem.,* **246**, 380-387.

Hochachka P. W. and Somero G. N., 2002: Temperature, in "*Biochemical Adaptation*", Oxford Univ. Press, New York, pp. 290-449.

Holmes K. C., Popp D., Gebhard W., and Kabsch W., 1990: Atomic model of the actin filament. *Nature*, **347**, 44-49.

Houk W. T. and Ue K., 1974: The measurement of actin concentration in solution: a comparison of methods. *Anal. Biochem.,* **62**, 66-74.

Hu M. C., Sharp S. B., and Davidson N., 1986: The complete sequence of the mouse skeletal alpha-actin gene reveals several conserved and inverted repeat sequences outside of the protein-coding region. *Mol. Cell. Biol.,* **6**, 15-25.

Hurley J. H., 1996: The sugar kinase/heat shock protein 70/actin superfamily: Implications of conserved structure for mechanism. *Annu. Rev. Biomol. Struct.,* **25**, 137-162.

Imamura S., Ojima N., and Yamashita M., 2003: Cold-inducible expression of the cell division cycle gene CDC48 and its promotion of cell proliferation during cold acclimation in zebrafish cells. *FEBS Lett.,* **549**, 14-20.

Itoi S., Kinoshita S., Kikuchi K., and Watabe, S., 2003: Changes of carp F0F1-ATPase in association with temperature acclimation. *Am. J. Physiol. Regul. Integr. Comp. Physiol.,* **284**, R153-R163.

Ikkai T. and Ooi T., 1966: The effects of pressure on F-G transformation of actin. *Biochemistry,* **5**, 1551-1560.

Iwamoto T. and Stein D. L., 1974: A systematic review of the rattail fishes (Macrouridae: Gadiformes) from Oregon and adjacent waters. *Occ. Pap. California Acad. Sci.,* **111**, 1-79.

Iwamoto T. and Sazonov, Y. I., 1988: A review of the southeastern Pacific *Coryphaenoides* (sensu lato) (Pisces, Gadiformes, Macrouridae). *Proc. California Acad. Sci.,* **45**, 35-82.

Jaenicke R., 1983: Biochemical processes under high hydrostatic pressure. *Naturwissenschaften,* **70**, 332-341.

Kabsch W., Mannherz H. G., Suck D., Pai E. F., and Holmes K. C., 1990: Atomic structure of the actin:DNase I complex. *Nature,* **347**, 37-44.

Khaitlina S. Y., Moraczewska J., and Strzelecka-Golaszewska H., 1993: The actin/actin interactions involving the N-terminus of the DNase-I-binding loop are crucial for stabilization of the actin filament. *Eur. J. Biochem.,* **218**, 911-920.

Kikuchi K., Itoi S., and Watabe S., 1990: Increased levels of mitochondorial ATP synthase ?-subunit in fast skeletal muscle of carp acclimated to cold temperature. *Fisheries Sci.,* **64**, 629-636.

Kimura M., 1980: A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.,* **16**, 111-120.

Kinosian H. J., Selden L. A., Estes J. E., and Gershman L. C., 1991: Thermodynamics of actin polymerization; influence of the tightly bound divalent cation and nucleotide. *Biochim. Biophys. Acta,* **1077**, 151-158.

Kinosian H. J., Selden L. A., Estes J. E., and Gershman L. C., 1993: Nucleotide binding to actin. Cation dependence of nucleotide dissociation and exchange rates. *J. Biol. Chem.,* **268**, 8683-8691.

Kimura M., 1968: Evolutionary rate at the molecular level. *Nature,* **217**, 624-626.

Kocher T. D., Thomas W. K., Meyer A., Edward S. V., Paabo S., Villablanca F. X., and Wilson A. C., 1989: Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proc. Natl. Acad. Sci. USA,* **86**, 6196-6200.

Krajewski C., Blacket M., Buckley L., and Westerman M., 1997: A multigene assessment of phylogenetic relationships within the dasyrid marsupial subfamily *Sminthopsinae*. *Mol. Phylogenet. Evol.,* 8, 236-248.

Kraulis P. J., 1991: MOLSCRIPT: a program to produce both detailed and schematic. plots of protein structures. *J. Appl. Crystallogr.,* **24**, 946-950.

Kraus F. and Miyamoto M. M., 1991: Rapid cladogenesis among the pecoran ruminants: evidence from mitochondrial DNA sequences. *Syst. Zool.,* **40**, 117-130.

Kuznetsova I. M., Yakusheva T. A., and Turoverov K. K., 1990: Contribution of separate tryptophan residues to intrinsic fluorescence of actin. Analysis of 3D *structure*. *FEBS Lett*. **452**, 205-210.

Lehrer S. S. and Kerwar, G., 1972: Intrinsic fluorescence of actin. *Biochemistry,* **11**, 1211-1217.

Levitt M., 1978: Conformational preferences of amino acids in globular proteins. *Biochemistry,* **17**, 4277-4285.

MacLean-Fletcher S. and Pollard T. D., 1980: Identification of a factor in conventional muscle actin preparations which inhibits actin filament self-association. Biochem. *Biophys. Res. Commun.,* **96**, 18-27.

Marshall N. B., 1979: Developments in the Deep-Sea Biology. Blandford Press, London, 566pp.

Martin A. P., Naylor G. J. P., and Palumbi S. R., 1992: Rates of mitochondrial DNA evolution in sharks are slow compared with mammals. *Nature,* **357**, 153-155.

Martin A. P. and Palumbi S. R., 1993: Body size, metabolic rate, generation time, and the molecular clock. *Proc. Natl. Acad. Sci. USA,* **90**, 4087-4091.

Merret N. R., 1978: On the identity and pelagic occurrence of larval and juvenile stages of rattail fishes (family Macrouridae) from 60° N, 20° W and 53° N, 20° W. *Deep See Res.,* **25**, 147-160.

Meyer A., Kocher T. D., Basasibwaki P., and Wilson, A. C., 1990: Monophyletic origin of Victoria cichlid fishes suggested by mitochondrial DNA sequences. *Nature,* **347**, 550-553.

Meyer A., 1992: Evolution of mitochondrial DNA in fish, in "*Biochemistry and Molecular Biology of Fishes*" (ed. by Hochachka, P. W., and Mommsen, T. P.), vol. 2, Elsevier Press, Amsterdam, pp. 1-38.

Mills E. M., 1983: Problems of deep-sea biology: an historical perspective, in "*Deep-Sea Biology*" (ed. by G. T. Rowe), John Wiley, New York, pp. 1-80.

Miya M. and Nishida M., 1996: Molecular phylogenetic perspective on the evolution of the deep-sea fish genus *Cyclothone* (Stomiiformes: Gonostomatidae). *Ichthyol. Res.,* **43**, 375-398.

Miya M. and Nishida M., 1998: Molecular phylogeny and evolution of the deep-sea fish genus *Sternoptyx*. *Mol. Phylogenet. Evol.,* **10**, 11-22.

Miya M., Kawaguchi A., and Nishida M., 2001: Mitogenomic exploration of higher teleostean phylogenies: a case study for moderate-scale evolutionary genomics with 38 newly-determined complete mitochondrial DNA sequences. *Mol. Biol. Evol.,* **18**, 1993-2009.

Miyamoto M. M. and Boyle S. M., 1989: The potential importance of mitochondrial DNA sequence data to eutherian mammal phylogeny, in "The Hierarchy of Life" (ed. by Fernholm B., Bremer K., Brundin L., and Jornvall H.), Elsevier Press,

Amsterdam, pp. 437-450.

Moraczewska J., Wawro B., Seguro K., and Strzelecka-Golaszewska H., 1999: Divalent cation-, nucleotide-, and polymerization-dependent changes in the conformation of subdomain 2 of actin. *Biophys. J.,* **77**, 373-385.

Morild E., 1981: The theory of pressure effects on enzymes. *Adv. Protein Chem.,* **34**, 93-166.

Mozhaev V. V., Heremans K., Frank J., Masson P., and Balny C., 1996: High pressure effects on protein structure and function. *Proteins,* **24**, 81-91.

Nakabo T., 2000: Macrouridae, in "*Fishes of Japan with Pictorial Keys to the Species*" (ed. by Nakabo T.), Tokai Univ. Press, Tokyo, pp. 417-435.

Normark B. B., McCune A. R., and Harrison R. G., 1991: Phylogenetic relationships of Neopterygian fishes, inferred from mitochondrial DNA sequences. *Mol. Biol. Evol.,* **8**, 819-834.

Ohno S., 1970: Evolution by Gene Duplication, Springer-Verlag, Heidelberg, 160pp.

Orlova A. and Egelman E. H., 1993: A conformational change in the actin subunit can change the flexibility of the actin filament. *J. Mol. Biol.,* **232**, 334-341.

Otterbein L. R., Graceffa P., and Dominguez, R., 2001: The crystal structure of uncomplexed actin in the ADP state. *Science,* **293**, 708-711.

Palumbi S. R. and Wilson A. C., 1990: Mitochondrial DNA diversity in the sea urchins, *Stronglyocentrotus purpuratus* and *S.droenachiensis*. *Evolution,* **44**, 403-415.

Perutz M.F., 1983: Species adaptation in a protein molecule. *Mol. Biol. Evol.,* **1**, 1-28.

Phleger C. F. and Laub R. J., 1975: Skeletal fatty acids in fish from different depths off Jamaica. Comp. *Biochem. Physiol.,* **94B**, 329-334.

Pollard T. D., 1986: Rate constants for the reactions of ATP- and ADP-actin with the ends of actin filaments. *J. Cell Biol.,* **103**, 2747-2754.

Pollard T. D. and Cooper J. A., 1986: Actin and actin-binding proteins. a critical evaluation of mechanisms and functions. *Annu. Rev. Biochem.,* **55**, 987-1035.

Rand D. M., 1994: Thermal habit, metabolic rate, and evolution of mitochondrial DNA. *Trends. Ecol. Evol.,* **9**, 125-131.

Reisler E., 1993: Actin molecular structure and function. *Curr. Opin. Cell Biol.,* **5**, 41-47.

Ritchie P. A., Bargelloni L., Meyer A., Taylor J. A., Macdonald J. A., and Lambert D. M., 1996: Mitochondrial phylogeny of Trematomid fishes (Nototheniidae, Perciformes) and the evolution of Antarctic fish. *Mol. Phylogenet. Evol.,* **5**, 383-390.

Saitou N. and Nei M., 1987: The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.,* **45**, 406-425.

Saunders P. M. and Fofonoff N. P., 1976: Conversion of pressure to depth in the ocean. *Deep-Sea Res.,* **23**, 109-111.

Sheterline P., Clayton J., and Sparrow J., 1999: *Actin* 4th ed., Oxford Univ. Press, New York, 272pp.

Siebenaller J. F. and Somero G. N., 1978: Pressure adaptive differences in lactate dehydrogenases of congeneric fishes living at different depths. *Science,* **201**, 255-257.

Siebenaller J. F. and Somero G. N., 1989: Biochemical adaptation to the deep sea. *Rev. Aquat. Sci.,* **1**, 1-25.

Siebenaller J. F., Somero G. N., and Haedrich R. L., 1982: Biochemical characteristics of macrourid fishes differing in their depths of distribution. *Biol. Bull.,* **163**, 240-249.

Siebenaller J. F., 1991: Pressure as an environmental variable: magnitude and mechanisms of perturbation, in "*Biochemistry and Molecular Biology of Fishes*" (ed. by Hochachka P. W. and Mommsen T. P.), vol. 1, Elsevier Press, Amsterdam, pp. 323-343.

Siebenaller J. F. and Murray T. F., 1995: The effects of pressure on G protein-coupled signal transduction, in "*Biochemistry and Molecular Biology of Fishes*" (ed. by Hochachka P. W. and Mommsen T. P.), vol. 5, Elsevier Press, Amsterdam, pp. 147-174.

Silva J. L. and Weber G., 1993: Pressure stability of proteins. *Annu. Rev. Phys. Chem.,* **44**, 89-113.

Smith K. L. Jr., 1978: Metabolism of the abyssopelagic rattail *Coryphaenoides armatus* measured in *situ*. *Nature,* **274**, 362-364.

Somero G. N., 1990: Life at low volume change: hydrostatic pressure as a selective factor in the aquatic environment. *Am. Zool.,* **30**, 123-135.

Somero G.N., 1992: Adaptations to high hydrostatic pressure. *Annu. Rev. Physiol.,* **54**, 557-577.

Somero G. N., 1995: Proteins and temperature. *Annu. Rev. Physiol.,* **57**, 43-68.

Springer M. S., Hollar L. J., and Burke A., 1995: Compensatory substitutions and the evolution of the mitochondrial 12 S rRNA gene in mammals. *Mol. Biol. Evol.,* **12**, 1138-1150.

Spudich J. A. and Watt S., 1971: The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.,* **246**, 4866-4871.

Stein D. L. and Pearcy W. G., 1982: Aspects of reproduction, early life history, and biology of macrourid fishes off Oregon, USA. *Deep-Sea Res.,* **29**, 1313-1329.

Steinar J. and Ingrid B., 1996: The complete mitochondrial DNA sequence of Atlantic cod (*Gadus morhua*): relevance to taxonomic studies among codfishes. *Mol. Mar. Biol. Biotechnol.,* **5**, 203-214.

Sturmbauer C. and Meyer A., 1992: Genetic divergence, speciation and morphological stasis in a lineage of African cichlid fishes. *Nature,* **358**, 578-581.

Stutz F. and Spohr G., 1986: Isolation and characterization of sarcomeric actin genes expressed in *Xenopus laevis embryos. J. Mol. Biol.,* **187**, 349-361.

Swezey R. R. and Somero G. N., 1982: Polymerization thermodynamics and structural stabilities of skeletal muscle actins from vertebrates adapted to different temperatures and hydrostatic pressures. *Biochemistry,* **21**, 4496-4503.

Swezey R.R. and Somero G.N., 1985: Pressure effects on actin self-assembly: interspecific differences in the equilibrium and kinetics of the G to F transformation. *Biochemistry,* **24**, 852-860.

Swofford D. L., 1998: Phyogenetic Analysis Using Parsimony (PAUP) 4d64 test version. Laboratory of Molecular Systematics MSC, MRC-534, Smithsonian Institution, Maryland.

Thompson J. D., Higgins D. G., and Gibson T. J., 1994: CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res.,* **22**, 4673-4680.

Tobacman L. S., Brenner S. L., and Korn, E. D., 1983: Effect of Acanthamoeba profilin on the pre-steady state kinetics of actin polymerization and on the concentration of F-actin at steady state. *J. Biol. Chem.,* **258**, 8806-8812.

Torigai M. and Konno K., 1997: Thermal stability of fish G-actin extracted from the acetone-dried myofibril-powder. *Fish. Sci.,* **63**, 403-406.

Uyeno T., 1967: A Miocene alepisauroid fish of a new family, *Polymerichthyidae*, from Japan. *Bull. Nat. Sci. Mus. Tokyo*, **10**, 383-391.

Uyeno T., 1980: On the rate of evolution in fishes. *Aquabiology*, **9**, 242-247 (in Japanese).

Venkatesh B., Tay B. H., Elgar G., and Brenner S., 1996: Isolation, characterization and evolution of nine pufferfish (*Fugu rubripes*) actin genes. *J. Mol. Biol.,* **259**, 655-665.

Vogler A. P. and Welsh A., 1997: Phylogeny of North American Cicindela tiger beetles inferred from multiple mitochondrial DNA sequenes. *Mol. Phylogenet. Evol.,* **8**, 225-235.

Waechter F. and Engel J., 1975: The kinetics of the exchange of G-actin-bound 1: N6-ethenoadenosine 5'-triphosphate with ATP as followed by fluorescence. *Eur. J. Biochem.,* **57**, 453-459.

Watabe S., Hirayama Y., Imai J., Kikuchi K., and Yamashita M., 1995: Sequences of cDNA clones encoding alpha-actin of carp and goldfish skeletal muscles. *Fish. Sci.,* **61**, 998-1003.

Watabe S., 2002: Temperature plasticity of contractile proteins in fish muscle. *J. Exp. Biol.,* **205**, 2231-2236.

Weber G. and Drickamer H. G., 1983: The effect of high pressure upon proteins and other biomolecules. *Q. Rev. Biophys.,* **16**, 89-112.

Wilson R. R. Jr., Siebenaller J. F., and Davis B. J., 1991: Phylogenetic analysis of species of three subgenera of *Coryphaenoides* (Teleostei :

Macrouridae) by peptide mapping of homologs of LDH-A4. *Biochem. Syst. Ecol.,* **18**, 565-572.

Wilson R. R. Jr., 1994: Interrelationships of the subgenera *Coryphaenoides* (Gadiforms: Macrouridae): Comparison of protein electrophoresis and peptide mapping. *Copeia,* **1**, 42-50.

Yamashita M., Ojima N., and Sakamoto T., 1996: Molecular cloning and cold-inducible gene expression of ferritin H subunit isoforms in rainbow trout cells. *J. Biol. Chem.,* **271**, 26908-26913.

Yancey P. H. and Siebenaller J. F., 1999: Trimethylamine oxide stabilizes teleost and mammalian lactate dehydrogenases against inactivation by hydrostatic pressure and trypsonolysis. *J. Exp. Biol.,* **202**, 3597-3603.

Yancey P. H., Fyfe-Johnson A.L., Kelly R.H., Walker V.P., and Aunon M.T., 2001: Trimethylamine oxide counteracts effects of hydrostatic pressure on proteins of deep-sea teleosts. *J. Exp. Zool.,* **289**, 172-176.

Yancey P. H., Blake W.R., and Conley J., 2002: Unusual organic osmolytes in deep-sea animals: adaptations to hydrostatic pressure and other perturbants. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.,* **133**, 667-676.