

cDNA Cloning of Paramyosin from Several Kinds of Squid Mantle Muscle

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Abstract

Paramyosin is a rod-shaped muscle protein found exclusively in invertebrates, with α -helices coiled around each other to form a coiled-coil structure. Marine organisms in which the primary structure of paramyosin has been determined are mollusks, including abalone (*Haliotis discus*), mussels (*Mytilus galloprovincialis*), octopus (*Octopus bimaculoides*), and oyster (*Crassostrea gigas*). In contrast, the primary structure of squid paramyosin, which is of particular interest, has yet to be reported. In the present study, cDNA cloning of paramyosins from four squid species, the neon flying squid (*Ommastrephes bartramii*), the Humboldt squid (*Dosidicus gigas*), the golden cuttlefish (*Sepia esculenta*), and the clawed armhook squid (*Gonatus onyx*), was performed to determine the following: the 2605-bp *O. bartramii* paramyosin gene containing a 2574-bp open reading frame (ORF), the 2691-bp *D. gigas* paramyosin gene containing a 2640-bp ORF, the 2631-bp *S. esculenta* paramyosin gene containing a 2574 ORF, and the 2609-bp *G. onyx* paramyosin gene containing a 2574-bp ORF. The primary structure of the four squid paramyosins was found to contain heptad repeats and an ACD (assembly competence domain), which are characteristic of a coiled coil. A phylogenetic analysis was performed with paramyosin sequences from species including the four squid species examined in this study, the results of which indicated that the four squid paramyosins form a group independent from the paramyosins of other species, to which octopus paramyosins are closest.

Keywords

Paramyosin, Squid, cDNA Cloning, Mantle Muscle, Phylogenetic Analysis

1. Introduction

Paramyosin is a protein found exclusively in invertebrate muscles [1] [2]. Para-

myosin molecules polymerize into a core structure, surrounding which myosin binds to form a thick filament that is considered to function similarly to vertebrate thick filaments [3]. Paramyosin constitutes 5% to 50% of invertebrate muscles depending on the species and the body part [4]. Under physiological conditions, α -helices with a molecular weight of approximately 100,000 coil around each other to form a coiled-coil structure [5] [6], the primary structure of which contains a heptad repeat, a characteristic repeating pattern of seven amino acid residues represented as *abcdefg* [7]. The hydrophobic interactions between the *a* and *d* residues as well as the electrostatic interactions between the *e* and *g* residues in the paramyosin molecules confer stability to the resulting dimer.

Squid musculature consists of obliquely striated muscles that are different from vertebrate striated muscles [8]. Apart from mollusks, obliquely striated muscles are found in nematodes, annelids, brachiopods, and chaetognaths. Obliquely striated muscles are referred to as such due to their structure in which each filament is slightly displaced relative to the next, so that adjacent sarcomeres are arranged in an oblique pattern. The muscle fibers are further arranged helically to form spindle-shaped muscle cells.

Squid muscles are eaten in raw or cooked. As the muscle of squid is covered by four layers of skin and is tough in texture when eaten in raw, it is often prepared by scoring the surface with a knife [9]. It can also be highly processed into a product that is similar to fish paste, so that it is more soft and easier to chew for people of all ages. However, the processing of squid meat is unique to that of fish meat in two aspects. One is that the gel strength and the elasticity do not increase when it is left to settle, in which highly active proteases present in squid meat are believed to be involved [10] [11]. When squid meat is minced, the endogenous proteases are activated to cause the proteolysis of muscle proteins that are essential in thermal gelation, resulting in poor gel formation. The other aspect is the unique texture of squid paste compared to that of fish paste. This difference in texture has been suggested to be attributable to paramyosins present in invertebrate meats. It has also been reported that paramyosin purified from scallop adductor muscle has a high gel-forming ability [12]. The muscle-processing property also varies among species of squid, some of which are more suitable for sashimi and others for processed foods. It has not been clear what factor contributes their differences. The differences in texture compared to other molluscan species such as shellfish, as well as among species of squid, probably also result from differences in the properties of muscle proteins, which led us to focus on paramyosin.

Marine organisms in which the primary structure of paramyosin has been determined are mollusks, including abalone (*Haliotis discus*, Accession number: BAJ61596), mussels (*Mytilus galloprovincialis*, O96064), octopus (*Octopus bimaculoides*, XP-014783284), oyster (*Crassostrea gigas*, XP-011429255), scallops (*Mizuhopecten yessoensis*, XP-021345467), and a species of Lingula (*Lingula anatina*, XP-013380361). With regards to squid paramyosins, the amino acid

composition has been described in the Japanese flying squid (*Todarodes pacificus*) [13], although the primary structure, which is of particular interest, has yet to be reported. The determination of the primary structures of squid paramyosin is possibly available for understanding the reason of the species-specific food processing. Thus, the present study was conducted with the aim of determining the primary structure of paramyosins from the mantles of edible squid species, including three species in the order Teuthida and one species in the order Sepiida, as well as characterizing the squid paramyosins.

2. Materials

Frozen mantle muscle from four squid species, *O. bartramii*, *D. gigas*, *S. esculenta*, and *G. onyx*, were purchased from IDO-SYOTEN Company (Iwate, Japan). The frozen mantle muscles were stored at -60°C until use.

3. Methods

3.1. cDNA Cloning

Initially, total RNA was prepared from 80 mg of the mantle muscle of each species using 0.8 ml ISOGEN II solution (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. cDNA was synthesized using total RNA and oligo dT primers with a reverse transcriptase (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. cDNA synthesis parameters were as follows: 42°C for 60 min, followed 70°C for 15 min. Primers were designed from the conserved sequences of the paramyosins of oyster, mussels, abalone, and octopus previously reported. PCR, 3' RACE, and 5' RACE methods were performed to obtain a DNA fragment, and this fragment was then sequenced. PCR for first-strand cDNA synthesis was performed using a Go Taq Master Mix (Promega Corporation, Tokyo, Japan). PCR parameters for the first PCR were as follows: Initial denaturation at 95°C for 30 s, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. Nested PCR was performed using the same PCR parameters, except that samples of the first PCR products were 10-fold diluted. PCR parameters for the 3' RACE analyses were as follows: Initial denaturation at 95°C for 30 s, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The 5' RACE analyses were performed using kits provided by Invitrogen (Carlsbad, CA) according to the manufacturer's instructions. PCR for full-length cDNA cloning was performed using platinum[®] Pfx Taq DNA polymerase (Invitrogen). The full-length PCR parameters were as follows: 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 51.5°C for 30 s, and 72°C for 3 min. DNA analysis was performed using BigDye Terminator v3.1 (Thermo Fisher Scientific, Kanagawa, Japan).

3.2. SDS-PAGE

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by using 12.5% commercial polyacrylamide gels (e-PAGEL, ATTO

Corporation, Tokyo, Japan) according to the method of [14]. Mantle muscle (0.2 g) and protein dissolution solution (3.75 μ l) of 20 mM Tris-HCl (pH 8.0)-2% SDS-8 M urea-2% β -mercaptoethanol were mixed and boiled for 2 min. And then muscle was dissolved by stirring overnight. Prestained molecular weight markers (Bio-rad, Hercules, CA) were used as standard. The gel was stained with Coomassie Brilliant Blue (CBB) R-250 after electrophoresis for 1.5 hours and then destained overnight with 30% methanol containing 10% acetic acid.

3.3. Amino Acid Sequence of Four Paramyosin

The polyacrylamide gel applied to 1/50 (v/v) samples to CBB stained one was stained with AE-1360 Ez Stain Silver (ATTO, Tokyo, Japan). A gel slice was cut into small pieces and destained with destaining solution (15 mM $K_3 [Fe(CN)_6]$, 50 mM $Na_2S_2O_3$). Destained gel pieces were trypsinized as described in the manual for the In-Gel Tryptic Digestion Kit (Thermo Scientific, Waltham, MA). The peptide mixtures obtained were subjected to a nanoscale liquid chromatography-electrospray (Thermo Scientific) equipped with a captive spray ionization source (Michrom Bioresources, Auburn, CA) and an Advance UHPLC System (Michrom Bioresources).

3.4. Phylogenetic Tree Analysis

The deduced amino acid sequences were aligned using the ClustalW program (EMBL-EBI: The European Bioinformatics Institute, European Molecular Biology Laboratory). Subsequently, a phylogenetic tree was constructed based on the neighbor-joining method using software Mega6 software [15].

4. Results and Discussion

4.1. cDNA Cloning of Paramyosins from Four Squid Species

Initially, cDNA was synthesized from frozen muscle RNA. Using this cDNA as a template, PCR was performed with the primers (pmF-1,2 and pmR-1,2) designed from paramyosin sequences of oyster, octopus, abalone, and mussels, as previously reported, to obtain the internal sequence of squid paramyosins (Figure 1, Table 1). The obtained sequences were then used to design the internal primer for amplification of the 5' end and the 3' RACE primer to obtain the partial sequence. For *S. esculenta* (Figure 1(c)), 5' RACE was attempted, in which the partial sequence containing the start codon was successfully amplified. The obtained N-terminal untranslated region of *S. esculenta* paramyosin was then used to design the forward primer (pm5'-F1) and the reverse primers (pm5'-R1,2) for full-length amplification. Using the designed primers for full-length amplification, PCR was performed for the amplification of the whole length of the *S. esculenta* paramyosin sequence. The primers for full-length amplification were subsequently used for amplification of the whole paramyosin sequence of *O. bartramii*, *D. gigas*, and *G. onyx*.

The full-length amplified fragments were inserted into the T-easy vector

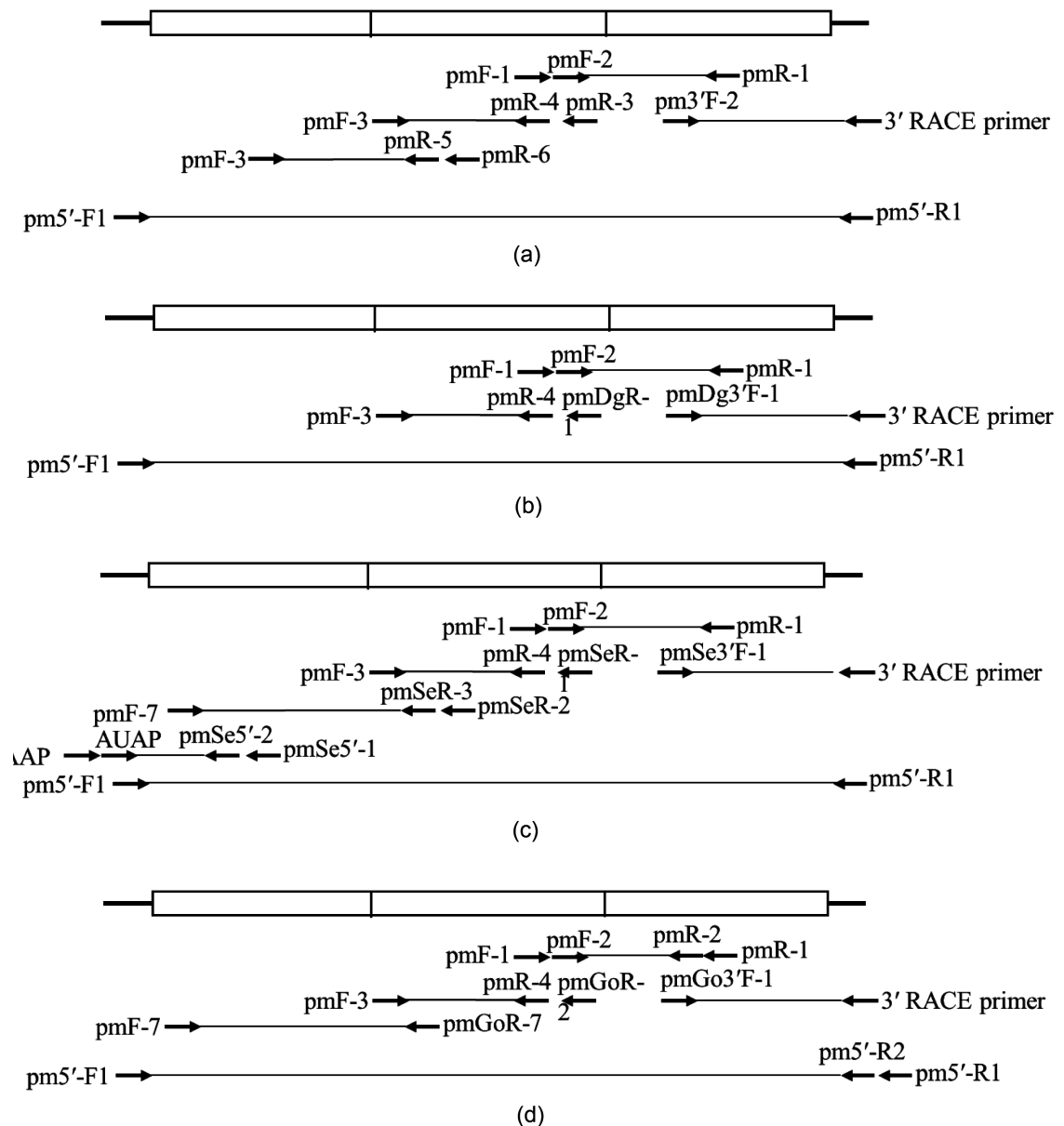


Figure 1. Schematic representation of the PCR strategy used for the full-length cloning of four squid paramyosins. (a) *Ommastrephes bartramii*. (b) *Dosidicus gigas*. (c) *Sepia esculenta*. (d) *Gonatus onyx*.

(Promega Corporation) for cloning. The entire base sequence of paramyosin was determined through sequence analysis for these clones. The *O. bartramii* paramyosin gene was cloned as a 2605-bp fragment containing a 2574-bp ORF, encoding a polypeptide of 858 amino acids. The *D. gigas* paramyosin gene was cloned as a 2691-bp fragment containing a 2640-bp ORF, encoding a polypeptide of 880 amino acids. The *G. onyx* paramyosin gene was cloned as a 2609-bp fragment containing a 2574-bp ORF, encoding a polypeptide of 858 amino acids. The *S. esculenta* paramyosin gene was cloned as a 2631-bp fragment containing a 2574 ORF, encoding a polypeptide of 858 amino acids. The entire base sequence and the deduced amino acid sequence obtained for *O. bartramii*, *D. gigas*,

Table 1. The sequences of primers shown in **Figure 1**.

Primer name	Sequence	Primer name	Sequence
3' end		cDNA cloning	
pm3 F-2	AAGTCTGCGCAAGCAGCTTG	pmF-1	GNYTNMGNGARAARGAYGARGA
pmDg3 F-1	TCGGAGATGAACTCAGACAGG	pmF-2	AARAARTAYGARACNGAYATHMG
pmSe3 F-1	CAACCGACTTTCCGATGAACTC	pmR-1	AANGCYTCNGCYTCYTCNARNC
pmGo3 F-1	GTTGAGGCCAACAGACTTTCTG	pmR-2	TTNCKNARNWSYTCNGCYTTYTT
3'RACE primer	CTGTGAATGCTGCGACTACGAT	pmF-3	GAYAAYYTNAAYGCNMGNTAYG
5' end		pmR-3	CGTTGAGTCTGTTCCGTCTAT
pmSe5 ⁻¹	TCTCTACGATTGAGCTCGC	pmR-4	TCYTCRTCYTTYTCNCKNARNC
pmSe5 ⁻²	TTCTGCAACGAGTTTCTCGG	pmF-4	CARYTNATHATHGARATHGA
AAP	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG	pmR-5	TTGCTAAGTTGGTTACGC
AUAP	GGCCACGCGTCGACTAGTAC	pmR-6	GTTACTTTCGGATTCCCTC
Full-length		pmF-7	NATHMGNGARYTNGARGAYG
pm5 ⁻¹ F1	CTTTCGGTATCACACTGC	pmDgR-1	AGGCTAGTTTCCACTTCC
pm5 ⁻¹ R1	GCTGCTCTATTGCGATCA	pmSeR-1	CATTCTGTCGATTGGCAGTATC
pm5 ⁻¹ R2	CTGCTCTATTGTGATCAGTG	pmSeR-2	ATTTATACGGACTCGTGC
		pmSeR-3	CATTCATTCTGCGTCTGAG
		pmGoR-2	TCAAGAGCACTCTCAAGTTC
		pmGoR-7	ATCTTGCTAAGTTGGGTACG

S. esculenta, and *G. onyx* paramyosins were submitted to the DNA Data Bank of Japan (DDBJ) (Accession numbers: LC272578, LC272579, LC272580, and LC272083).

In general, paramyosin forms a coiled coil structure that contains a heptad repeat consisting of hydrophobic amino acids and a 28-residue repeat consisting of charged amino acids [2] [6] [16] [17] [18]. In the C-terminal region, it also contains an assembly competence domain (ACD) consisting of 29 residues [19], through which the paramyosin molecules are believed to polymerize. Heptad repeats were identified throughout almost the entire sequences of the four squid paramyosins (**Figures 2-5**), in which hydrophobic amino acids were found in many of the *a* and *d* positions [20] [21] and charged amino acids were found in the *e* and *g* positions, displaying a structure characteristic of a coiled coil. Skip residues were also identified, resulting in the modification of heptad repeats into octad repeats. The skip residues further contribute to form 28-residue repeats containing repeated sequences [21] [22]. As shown in the figures, almost the entire region of the paramyosin sequences, excluding parts of the N- and C-terminal regions, was identified to form a coiled-coil structure. The sequences were also found to end in C-terminal tail pieces.

The percentage similarity among paramyosin sequences was found to be 96% between *O. bartramii* and *D. gigas*, 88% between *O. bartramii* and *G. onyx*, 90% between *O. bartramii* and *S. esculenta*, 86% between *D. gigas* and *G. onyx*, 87% between *D. gigas* and *S. esculenta*, and 89% between *G. onyx* and *S. esculenta*

<i>d e f g a b c d e f g a b c d e f g a b c d e f g a b c</i>	
ctttgaacgaatcaacagaataca	24
atggactacgattccgaogtgaagattacgcgctccgctcgccgttatgaggtgtacagaggctct	90
M D Y D S D V K V T R S V R R Y E V Y R G S	22
tctcctgccaccagaaacaga ttggaggctagaatcagagag ctogaagatgcgatggatgcc gagaggacggcagaaatcagg	174
S P A T Q N R L E A R I R E L E D A M D A E R D G R I R	50
gocgaaaaactcgtttcagaa tacgttttccaactcgattct ttaagtgagcaactcgaagag gctggccttacttcgtaaca	258
A E K L V S E Y V F Q L D S L S E Q L E E A G L T S V T	78
cagagcgagctcagctgaag agggagtcagaagttaccaaa cttcgtaaagacatggaaactc ctcaatgttcaacacgaaagttct	345
Q S E L S R K R E S E V T K L R K D M E L L N V Q H E S S	107
gaacacagcatgaggaagcgt catcaagagacatcaatgat ttatccgagcaggttattac atcacaacacagaagaacaga	429
E H S M R K R H Q E T I N D L S E Q V D Y I T K Q K N R	135
gttgaaaaagaaaatcacag cttctcgtggaattgacgga ttgcagacaagtttggatgta gtttcgaaagccaaggctgcc	513
V E K E K S Q L L V E I D G L Q T S L D V V S K A K A A	163
gccgaagctaaattggaagat atggatgccaagttggcaga cttaaagatcactgttgacag ttgacaacacacttacagat	597
A E A K L E G M D A Q V G R L K I T V D D L T K Q I T D	191
totaacctgtaagagctcgt ctcaccagaggaatttcgac taccagcaccaggtccaggag ctggatagcgtcaacgctgct	681
S N H V R A R L T Q E N F D Y Q H Q V Q E L D S A N A A	219
ttggctaagcctaagatgcag ttgcaggtcgaagcgaagac ttgaaacgttctctggatgat gaaactagtcgaagcgaac	765
L A K A K M Q L Q A Q A E D L K R S L D D E S R Q R A N	247
ttgagtgccagctctcagct ttgcaggtcacttcgataac ttgaaacgtcgtacagagg gaatccgaaagtaacagatc	849
L S A Q L S A L Q A D F D N L N A R Y E E S E S N S I	275
ctggctaaacacttagcaaa gtcaatgccgaatacaccgcc ctcaagaccgctatgaaaaa gaactctggccaagcagaggag	936
L R N Q L S K V N A E Y T A L K S R Y E K E L L A K T E	304
ttagaagatctcagacgccga atgaagctcagagttgctgaa ctgaagaccaacttgaacaa catcgtgtccgtatacaat	1020
L E D L R R R M N V R V A E L E D Q L E Q H R V R I N N	332
ctgaaaagactaagatcaaa ctcaccaccagatcaaaagaa cttaccattgaaattgaaat attcagattattgttcaagac	1104
L E K T K I K L T T E I K E L T I E I E N I Q I I V Q D	360
cttaccagaggaacgctcaa ctcgagaatgaaatcgttct ctgctcaaacgcattgaaaga ttgacaactgagaaaccaca	1188
L T K R N R Q L E N E N A S L L K R I E E L T T E N H N	388
cttgacgctgccaacaaagct cttgacaagacaaccatcgt ttgaaagttgttaaccgccaa ctcggtgaaagagtgataac	1272
L A A A N K A L E Q D N H R L K V V N A E L G R E V D N	416
cttaccagggaaaacaaacaa ctcactgacagcctccgtgag accactgctgcccttaagat gccaaccccaggtggctgaa	1356
L T R E N K Q L T D S L R E T T A A L K D A N R Q V A E	444
ttgcagccctgctgcccag ctcgagggccgaactgattct ctgtgtgctccctctgctgac actgaagagagcttgcgtgat	1440
L Q A L R A Q L E A E R D S L A V A L R D T E E S L R D	472
actgagaacaaactctatgct gctaccacagccctcaaccaa cttagagttgagatggagaa agactcctgaaaagatgaaaga	1527
T E N K L H A A T T A L N Q L R V E M E N R L R E K D E E	501
attgaaagcatcaggaagagc agcgcgccgctattgaaaga cttcagcgtactctctgtaa attgaaagcagatataagactgat	1614
I E S I R K S S A R A I E E L Q R T L V E I E A R Y K T E	530
atcagcagctgaaagaagat tatgagacagacatcagggaa ctcgaaaatgcccttgatact gccaaacagactcaacgctgaa	1698
I S R L K K K Y E T D I R E L E N A L N D L E N A L N A E	558
cacgtcaagttatcaaaagt ctcacatcagaaatcaggaa cttgaaaccagccttgaggaa gaaccgagaatctgtgaaagat	1782
H V K V I K S L H I R I K E L E T S L E E E R R I C E D	586
atcogctctcagctcaaccatc tctgaaagaaacgcatgtc ctcaccactgaacttgaagac tgcogtacacttttggaaacc	1866
I R S Q L T I S E R K R I V L T T E L E D C R T L L E T	614
gctgaacgtgcccgaacaaac gcogagaccgagctccagaa accacatccccttagcaga tgcacaactcctcctcacatct	1950
A E R A R K N A E T E L H E T T S R L S E C Q I L L T S	642
gtcaccatgacaagaacgt ttgaaactgacatccaggga atgcagagcagatcttgacgat gctattacaatatgctgccc	2034
V T N D K K R L E L D I Q G M Q S D L D D A I N N M R A	670
tccaacgacctgcccagataga ttgcaagttgaggtcaaccga ctcggcgatgaactcagacag gaactctgaaactacaagaac	2118
S N D R A D R L Q V E V N R L G D E L R Q E S E N Y K N	698
gocgaaagtctgcccgaagcag cttgaaatgaaatcagagaa atcaccatccgtttgaaaga gctgaaagttatgccagagggaa	2205
A E S L R K Q L E I E I R E I T I R L E E A E A Y A Q R E	727
ggaaaacatggttctctaaa ctcgaagcagatcagagac ttggaagcagagatagaagct gaacagcccgacaaggag	2289
G K R M V A K L Q A R I R D L E A E Y E A E Q R R T R E	755
gctattgccattagccgcaag aatgaacgcagcttgaaggaa ctcaccgcagttgttgaggat gaaccgccatcatgagcgaa	2373
A I A I S R K N E R S L K E L T A V V E D E R R I M S E	783
cttaccagogccaacgaacag atgttcttaagatgaagacc tacaacgcagatcagggaa gccgaaagattgctacatc	2457
L T S A N E Q M F L K M K T Y K R Q I E A E E V A T I	811
accatgaacaaataccgcaaa gccagagccttattgaaaga gctgatcacagggccgatatg gctgaaaagaatctcgtatct	2541
T M N K Y R K A Q S L I E E A D H R A D M A E K N L V S	839
atgcgcaggtctcgttccatgctcgtcaccgagagattacaaaagtgatcaaaatctaa	2601
M R R S R S M S V T R E I T K V I K I -	859
actc	2605

Figure 2. The complete base sequence and amino acid sequence of *Ommastrephes bartramii* paramyosin. The letters *d, e, f, g, a, b,* and *c* at the top indicate heptad repeats. Underlines indicate hydrophobic amino acids. Shaded letters indicate charged amino acids. Letters in bold indicate the ACD.

(Table 2). No clear difference was observed between the three species in the order Teuthida (*O. bartramii*, *D. gigas*, and *G. onyx*) and the one species in the order Sepiida (*S. esculenta*). The N-terminal region was found to be the region showing the least homology among the sequences of the four squid paramyosins. The amino acids in the ACD were highly conserved, showing an exact match between *O. bartramii* and *D. gigas*, and between *S. esculenta* and *G. onyx*. Only one-residue differences were observed among the sequences that did not show

d e f g a b c d e f g a b c d e f g a b c d e f g a b c

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atattattacatttccttgaacgaatccacagaaacaa          39
atggactacagattccgacgtgaaagttacgcctccgctgcccgcctatgaggtgtacagagctct          105
M D Y D S D V K V T R S V R R Y E V Y R G S          22
tctcctgccaccagaacaga ttggaggtgtttaaacaacgt cacatcacattogatacaacagaa ctagacagaaatggaatcacgg          192
S P A T Q N R L E V V K T R H I T F D Q T E L D R M E S R          51
tttogggctagaatcagagag ctogaagatcgatggatgcc gagagggacgcgagaatcagg gcogagaaactcgtttcagaa          276
E R A R I R E L E D A M D A E R D G R I R A E K L V S E          79
tacgtttfcaacfcgattct ttaagttagcaactcgaagag gctggccttactctcctaaca cagacggagctcagctgtaag          360
Y V F Q L D S L S E Q L E E A G L T S V T Q S E L S R K          107
agggagtcaagaattaccaaa cttcgtaaagacatggaactc ctcaacgttcaacacgaagttct gaacatagcatgaggaacgt          447
R E S E V T K L R K D M E L L N V Q H E S S E H S M R K R          136
catcaagagaccatcaatgat ttatccgagcaggttgattac atcacaaaaacagaagaacaga gttgaaaaaagaaatcacag          531
H Q E T I N D L S E E Q V D Y I T K Q K N R V E K E K S Q          164
cttctcgtgaaattgacgga ttgcagacaacttgatgta gtttogaagccaagctgcc ccogaagctaaattgaaagct          615
L V E I D G L Q T N L D V V S K A K A A A G E A K L E G          192
atggatgcccaagttggcaga cttagaatcaccttgtagcagac ttgacaaaaaacctgtcagac tctaaccatgaaagactcgt          699
M D A Q V G R L K I T V D D L T K Q L S D S N H V R A R          220
ctgaccaggagaatttcagac taccagcaccaggtccaggag ctgatagcgtactaactgctct ttggcaaaaggctaagatgcag          783
L T Q E N E D Y Q H Q V Q E L D S A N A A L M Q A K M Q          248
ttgcagcctcaagcccgaagc ttgaaactgtcttgatgat gaactagcgaacgcaccaac ttgagctccagctcactc          867
L Q A Q A E D L K R S L D D E S R Q R A N L S A A Q L S A          276
ttgcagcctgacttcgataac ttgaatgctcgtactcaggagc gaatccgaaagtaacagcatc ctgctgaacacacttagcaaa          951
L Q A D F D N L N A R Y E E E S E S N S I L R N Q L S K          304
gtcaatgccgaatacacagccc ctcaagagccgctatgaaaaa gaactcttgccaagacagaggag ttagaagatctcagacccga          1038
V N A E Y T A L K S R Y E K E L L A K T E E L E D L R R R          333
atgaacatcagaattgctgaa cttgaggaccacttgaacaa caccgtgtccgtatcaataat ctggaagactaagatcaaa          1122
M N I R I A E L E D Q L E Q H R V R I N N L E K T K I K          361
ctcaccacggagatcaagaa cttaccattgaaattgaaat attcagattattgttcaagac cttaccaagaggaacccgcaa          1206
L T T E I K E L T I R E I E N I Q I I V Q D L T K R N R Q          389
ctcgagaatgaaatgcttct ctgctcaaacgattgaggaa ttgactactgagaaccacaac cttgcagctcaacaaagct          1290
L E N E N A S L L K R I E E L T T E N H N L A S A N K A          417
cttgaacaagacaaccatcgt ttgaaggttgaatccgcaa ctccgtgaaagagtgataac cttaccagggaacaaacaa          1374
L E Q D N H R L K V V N A E L G E R V D N L T C R N K Q          445
ctcactgacagcctcogttag accactgctgcccctaaggat gctaaccgcccagtggtgaa ttgcagccctcgtgcccag          1458
L T D S L R E T T A A L K D A N R Q V A E L Q A L R A Q          473
ctcgaggcagaactgattct cttgocctcgccttctgtag actgaagagagcttgcgtgat actgagaacaaactccatct          1542
L E A E R D S L A V A L R D T E E S L R D T E N K L H A          501
gctaccacagcccttaaccaa ctcaaatgagatggagaaac agactccgtgaaaagatgaaaga attgaaagcatcaggaagagc          1629
A T T A L N Q L R I E M E N R L R E K D E E I E S I R K S          530
agcccccgcgtattgaaaga cttcagcgtactctcgttga attgaagccagatataagactgaa atcagcaggtgaaagaag          1716
S A R A I E E L Q R T L V E I E A R Y K T E I S R L K K K          559
tatgagacagacatcagggaa cttgaaaatgcccttgatct gccaacagactcaacgctgaa cacgtcaagactataagact          1800
Y E T D I R E L E N A L D T A N R L N A E H V K T I K T          587
ctgcacatcagaatcaaggaa gtggaactagccttgaggaa gaacgagaatctgtgaagat atccctccagctcaccatc          1884
L H I R I K E V E T S L E E E R R I C E D I R S Q G L T I          615
tctgaaagaaacgattgtc cttccactgaacttgaagac tgccgtacacttttggaaacc gctgaaactgcccgaagaat          1968
S E R K R I V L T T E L E D C R T L L E T A E R A R A R K N          643
gccgaaaccgagctccacgaa accacatccgccttagcgaa tgccaaatcctcctcacatct gtaccaatgacaagaacgt          2052
A E T E L H E T T S R L S E C Q I L L T S V T N D K K R          671
ttggaactgacatccaggga atgcagagcagatctgacgat gctatcaacaatatgctgct tctaagaccgtgcccagaga          2136
L E L D I Q G M Q S D L D D A I N N M R A S N D R A D R          699
ttgcaagttgaggtcaaccga ctcggagatgaactcagacag gaactgtgaaattacaagaac gctgaaagcctgcgcaagcag          2220
L Q V E V N R L G D E L R Q E S E N Y K N A E S L R K Q          727
cttgaattgaaatcagagaa ataccatcctgttggaaaga gctgaagcttatgcccagaggaa ggaaaacgactggtgtaag          2307
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L Q A R I R D L E A E Y E A E Q R R T R E A I A I S R K          784
aatgaaccagcttgaaggaa cttccagcttgttggat gaacccgcacatgagcgaa cttaccagcccacgaacag          2475
N E R S L K E L T A V V E D E R R I M S E L T S A N E Q          812
atgttcttaagatgaagacc tacaaacgacagatcgaggaa gccgaagaagtgtcacaatc accatgaacaataaccgcaaa          2559
M F L K M K T Y K R Q I E E A E E V A T I T M N K Y R K          840
gcccagagccttattgaaaga gctgatcacagggccgatag gctgaaaaaagatcctcatct          2622
A Q S L I E E A D H R A D M A E K N L A S          861
atggcaggtctgttccatgtgtgcaccgagagattacaaaagtgatcaaatctaa          2682
M R R S R S M S V T R E I T K V I K I -          881
actcaaaa          2691
    
```

Figure 3. The complete base sequence and amino acid sequence of *Dosidicus gigas* paramyosin. The letters *d*, *e*, *f*, *g*, *a*, *b*, and *c* at the top indicate heptad repeats. Underlines indicate hydrophobic amino acids. Shaded letters indicate charged amino acids. Letters in bold indicate the ACD.

an exact match.

4.2. Amino Acid Sequence of Four Paramyosin

SDS-PAGE analysis showed that four squid mantle muscles were constituted three major myofibrillar proteins; myosin heavy chain around 250 kDa, paramyosin around 90 kDa and actin around 40 kDa (Figure 6). The bands of paramyosin were subjected to the analysis for amino acid sequences after destained

d e f g a b c d e f g a b c d e f g a b c d e f g a b c

```

ttccttggatcgaatcaacagaatata
atggactacgattccgacgtgaagttacaogctccgctgctgotatgaggtgtacagaggcacc
M D Y D S D V K V T R S V R R Y E V Y R G T
tctcccactcagaaacaga ttggaggaaatcagagag ctogaagatgttcttgattcc gagaggacggcaggatcagg
S P T T Q N R L E A R I R E L E D V L D S E R D G R I R
gccgaaaactcgttcagaa tatactttccaacttgatgct gtcaacgagcagctcgaagag gctggtcttacttcggttaaca
A E K L V A E Y T F Q L D A V N E R L E E A G L T S V T
cagagcagctcaatogtaag agagagtotgaagtaataaa ctgcgaaagaccitgaactl attactgttcaacatgagagctct
Q S E L N R R K R E S E V N K L R K D L E L I T V Q H E S S
gaacaaagtatgaggaaact catcaagagacatcaatgat ttatccgaacaggttgattac atcacaacagaagaacaga
E Q S M R K R H Q E T I N D L S E Q V D Y I T K Q K N R
gttgaanaagaaaatcacag cttcttgggaaatgacgga ttgcagacaagcttagatgca gtttcgaaagccaaggctgcc
V E K E K S Q L V E I D G L Q T S L D A V S K A K A A
gtgaagcagattggaagga ttggagcccaagtggttagg ctgaagatcaccttagacgac ttgacgaaacactgacgat
A E G R L E G L D A Q V G R L K I T V D D L T K Q L A D
gccaaactgcaaaagctct ctaccagcagagaatttcgac taccagcaccaggtcaggag ctggatagcccaatgctgct
A N H A K A R L T Q E N F D Y Q H Q V Q E L D S A N A S
ttgcaaaagctaaatgcaat ttgcagactcaagcgaagac ttgaaacttctcttgatgat gaactagcgaacgcatcaac
L A K A K M Q L Q T Q A E D L K R S L D D E S R Q I N
ttgactgccaattttcggca ctgcagcgcacttggataat ttgaacgcccctacaggaa gaactgaaagtaataactaca
L T A Q F S A L Q A D F D N L N A R Y E E S E S N T T
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L R N Q L S K I N T E Y S A L K S R Y D K E L M A K T E E
ttagaagatctcagacgcaga atgaatccagagttgctgaa ctggagcacaacttgaacaa gcacagctcagataaataat
L E D L R R R M N A R V A E L E D Q L A E Q A R V R I N N
ctggaaaagacaagatcaag ctaccactgagatgaagaa ctcaaatgaaatgaaact attcagatttgcacaagac
L E K T K I K L T T E I K E L T M E I E N I Q I I V Q D
cttactaagaaaatcgtcaa ctggagacgagaatgcttct ttgctcaaacgttggatgag ttgactcagaaaatgctgac
L T K R N R Q L E N E N A S L L K R V D E L T S E N A S
ttgcaagcccaacaaagcc ctgaaacagaacacatcgc ttgaaacttctcagatgca ctggagcagagatgataac
L S A A N K A L E Q D N H R L K V A N A E L G E R V D N
cttaccagagaaaacaagcaa ctacagacaactctctgtag acaactactgcccttaaggat gtaaccccaagtagctgag
L T R E N K Q L T D N L R E T T T A L K D A N R Q V A E
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L Q A L R A Q L E A E R D S L A S A L R D T E E S L R D
gctgaaaacagctcctcagct gccaccacagctcctcaaccaa ctgagggctgagatggaacaa agactcctgaaaagatgaaaga
A E N K L H A A T T A L N Q L R A E M E N R L R E K D E E
attgaaagcagcagaagagc agtctctgcaattgaaaga ctccagcgcactctcattgaa gtcgaaacaagatacaagctgaa
I E S I R K S S A R A I E E L Q R T L I E V E T R Y K S E
atcaacagccttaagaagaag tatgaaactgacatcagagaa ctggagaacgcccctgatact gccanrgacagaatgctgaa
I N R L K K K Y E T D I R E L E N A L D T A N R K N A S E
catgtcaagttatcaagctc ttgcacatcagaaatcaagaa ctgaaacagccttgaagag gaacgccatttctgagat
H V K V I K S L H I R I K E L E T S L E E E R R I S E D
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I R G Q L S I S E R K R I A I T T E L E D C R S L L E T
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I T N E K K R L E L D I Q G M Q S D L D D A I N N M R A
gccaatgaccgcgtgacaga ctggctgttgaggtoaacgaa ctttccgatgaactcaagcag gaactcgaactacaagaat
A N D R A D R L A V E V N R L S D E L K Q E S E N Y K N
gctgaaagtttgcgcaagcag ctgaaaattgagatcagggaa attaccatccgttagaggaa gctgaagccttgcgcaagaaga
A E S L R K Q L E I E I R E I T I R L E E A E A Y A Q R E
ggaaaacaaatgctgcaaaa ttgcagcgcgggtaagagat ttggaaggagagctgaaagt gaacgccgcaacacagag
G K R M V A K L Q A R V R D L E G E L E S E Q R R T R E
gcaatcacatataacgtaag aatgaacgcaatttgaaggaa ctactgtagtgttgaagac gaacgccatcatgagcga
A I T Y N R K N E R N L K E L T V V V E D E R R I M S E
cttactagcccaacgaacag atgttcttaaatgaggacc tacaacgacagatcaggaa gctgaaagattgctacaata
L T S A N E Q M F L K M R T Y K R Q I E E A E E V A T I
accatgaacaaataccgtaag gccagcagcttatogaagaa gctgaacacagggctgatag gctgaaaagaactttcagca
T M N K Y R K A Q Q L I E E A E H R A D M A E K N L S A
atggcaggtctcgtcctatgtctgcccagagatcaccaaagtggtcaactctaa
M R R S R S M S V T R E I T K V V K L -
acgttgactgatcgaatagagcagc

```

Figure 4. The complete base sequence and amino acid sequence of *Sepia esculenta* paramyosin. The letters *d*, *e*, *f*, *g*, *a*, *b*, and *c* at the top indicate heptad repeats. Underlines indicate hydrophobic amino acids. Shaded letters indicate charged amino acids. Letters in bold indicate the ACD.

and trypsinized. As an example, deduced amino acid sequence of *O. bartramii* paramyosin and the covered regions of the peptide fragments was shown in **Figure 7**. The peptide fragments exhibited high coverage of 56.18% (484 residues). In addition, either end the trypsinized sites was K or R. Therefore, the trypsin treatment was identified as successful. The other three squids, *D. gigas*, *S. esculenta* and *G. onyx* also showed high coverage of 46.36% (407 residues), 68.53% (587 residues) and 43.47% (372 residues), respectively (date not shown). From these results, it was suggested that paramyosin genes determined in this

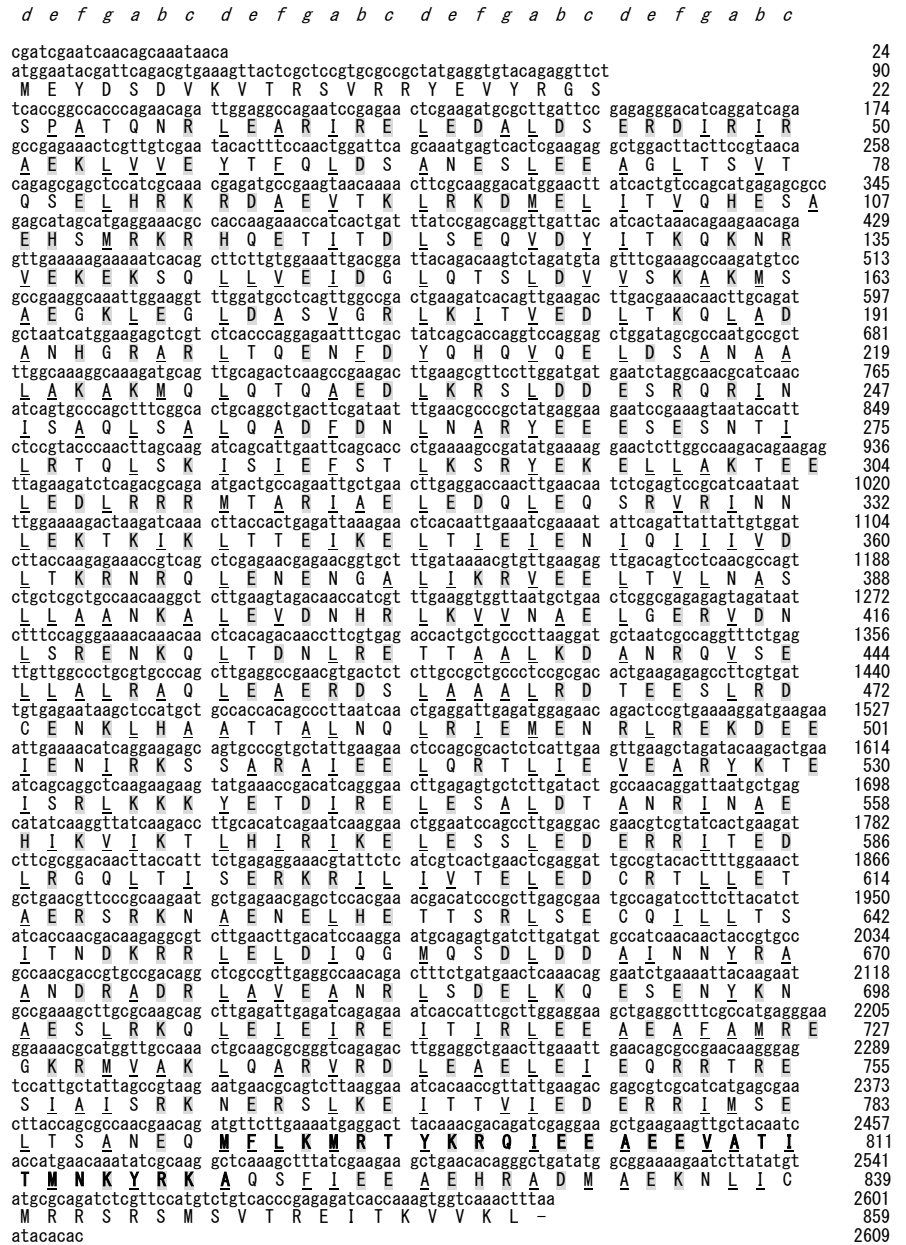


Figure 5. The complete base sequence and amino acid sequence of *Gonatus onyx* paramyosin. The letters *d, e, f, g, a, b,* and *c* at the top indicate heptad repeats. Underlines indicate hydrophobic amino acids. Shaded letters indicate charged amino acids. Letters in bold indicate the ACD.

study was actually expressed in each mantle muscles and existed as protein.

4.3. Phylogenetic Analysis

Phylogenetic analysis of paramyosin had been reported in fluke species and tapeworm species [23] [24]. An unrooted phylogenetic tree was constructed using a total of 26 paramyosin sequences, including one sequence from one octopus species, 13 sequences from seven shellfish species, five sequences from five fluke species, and three sequences from three tapeworm species, which are all known,

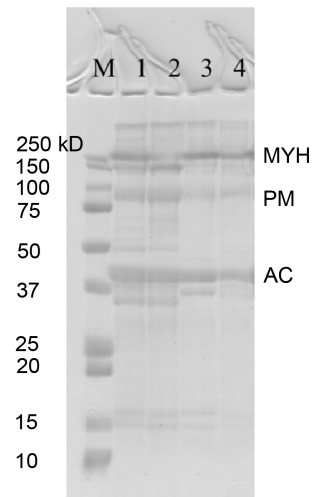


Figure 6. SDS-PAGE analysis of four squid mantle muscles. M: Molecular weight marker, MYH: Myosin heavy chain, PM: Paramyosin, AC: Actin. Lane 1 *Ommastrephes bartramii*, Lane 2 *Dosidicus gigas*, Lane 3 *Sepia esculenta*, Lane 4 *Gonatus onyx*.

MDYDSVKVTRSVRRYEVYRGSSPATQNRLEARIRELEDAMDAERDGRIRAEKLVSEYVFQDLSLSEQLLEAGL
 TSVTQSELSEKRESEVTKLRKDMELLNVOHESSEHSMRKRHQETINDLSEQVDYITKQKNRVEKEKSQLLVEID
 GLQTSLDVVS KAKAAAAEAKLEGMDAQVGR LKITVDDLTKQLTDSNHVRLTQENFDYQHQQVQELDSANAALAK
 AKMQLQAQAE DLKRS LDDSRQRANLSAQLSALQADFDNLNARYEESESENSILRNQLSKVNAEYTKLSRYEK
 ELLAKTEELEDLRRRMNVVRAELEDQLEQHRVRIINLEKTKIKLTTTEIKELTIEIENIQIIVQDLTKRNRQLEN
 ENASLLKRIEELTTENHNLAANKALEQDNHRLKVVNAELGERVDNLTRENKQLTDSLRETTAALKDANRQVAE
 LQALRAQLEAERDSLAVALRDTESLRDTENKLAATTALNQLRVEMENRLREKDEETESIRKSSARATEELQR
 TLVEIEARYKTEISRLLKKYETDIRLEENALDTANRLNAEHVKVIKSLHIRIKELETSLLEEERRICEDIRSQLT
 ISERKRIVLTTELED CRTLLETAERARKNAETELHETTSRLSECQILLTSVTNDKRLLELDIQGMQSDLLDAIN
 NMRASNDRADRLQVEVNR LGDEL RQES ENYKNAESLRKQLEIETIREITIRLEEA EAYA QREGKRMVAKLQARIR
 DLEAEYEAQRRTREAIATSRKNERSL KELTAVVEDERRIMSELTSANEQMF LKMKTYKRQIEEAEEVATITMN
 KYRKAQSLIEADHRADMAEKNLVSMRRSRMSVTR EITKVIKI

Figure 7. Deduced amino acid sequence of *Ommastrephes bartramii* paramyosin. Out-line characters sequences show matching with the peptide fragments of the separated and trypsinized paramyosin of *O. bartramii* (coverage: 56.18%, 482 residues).

Table 2. Similarities among four squid paramyosins (%).

	<i>O. bartramii</i>	<i>D. gigas</i>	<i>S. esculenta</i>	<i>G. onyx</i>
<i>O. bartramii</i>	-	96	90	88
<i>D. gigas</i>	96	-	87	86
<i>S. esculenta</i>	90	87	-	89
<i>G. onyx</i>	88	86	89	-

in addition to the sequences from the four squid species determined in this study, in order to examine the characteristics of the paramyosin structure (Figure 8, Table 3). The four squid paramyosins formed a group independent from the paramyosins of other species, to which the octopus paramyosin was most closely positioned. Fluke and tapeworm species also formed a group, whereas shellfish species remained dispersed. Shellfish species vary in shape such as univalves and bivalves, which may form independent groups. Nonetheless, these

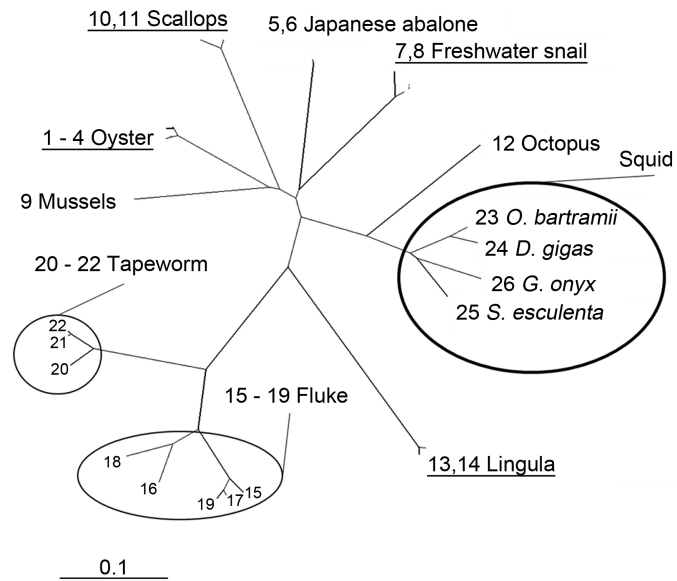


Figure 8. Phylogenetic analysis of several mollusk paramyosins. The numbers correspond to the species in **Table 3**.

Table 3. Species used for phylogenetic analysis.

number	species name	number	species name
1	<i>Crassostrea gigas</i> ₁	14	<i>Lingula anatina</i> ₂
2	<i>Crassostrea gigas</i> ₂	15	<i>Schistosoma japonicum</i>
3	<i>Crassostrea gigas</i> ₃	16	<i>Clonorchis sinensis</i>
4	<i>Crassostrea gigas</i> ₄	17	<i>Schistosoma haematobium</i>
5	<i>Haliotis discus discus</i>	18	<i>Paragonimus westermani</i>
6	<i>Haliotis discus hannai</i>	19	<i>Schistosoma mansoni</i>
7	<i>Biomphalaria glabrata</i> ₁	20	<i>Echinococcus granulosus</i>
8	<i>Biomphalaria glabrata</i> ₂	21	<i>Taenia saginata</i>
9	<i>Mytilus galloprovincialis</i>	22	<i>Taenia solium</i>
10	<i>Mizuhopecten yessoensis</i> ₁	23	<i>Ommastrephes bartramii</i>
11	<i>Mizuhopecten yessoensis</i> ₂	24	<i>Dosidicus gigas</i>
12	<i>Octopus bimaculoides</i>	25	<i>Sepia esculenta</i>
13	<i>Lingula anatina</i> ₁	26	<i>Gonatus onyx</i>

shellfish species showed greater diversity compared to the other groups of species. Fluke [25] paramyosins showed high sequence similarities within the group, with percentages of 93% or greater. Tapeworm [26] [27] paramyosins also showed high sequence similarities with percentages of 98% or greater. Shellfish paramyosins showed lower sequence similarities compared to other groups of species, with percentages from 71% to 86%. The underlined species in **Figure 6** have multiple isoforms of paramyosin, and the respective isoforms were found close to each other. For instance, oyster is known to have four paramyosin iso-

forms, which all have high similarities to each other with percentages of 96% or greater. Similarly, paramyosin isoforms from two species of fresh water snail, two species of scallops, and two species of *Lingula*, whose sequences are registered in the database, have high similarities among their respective isoforms, with percentages exceeding 96%. For these species that are known to have multiple isoforms, the sequences were suggested to encode paramyosin genes through genome analyses. In contrast, in species whose paramyosin sequences have been determined by means of cDNA cloning, such as those in the present study (including *O. bartramii*, *D. gigas*, *S. esculenta*, *G. onyx*, and mussel species), only a single gene has been identified. Although genome databases collect the sequences of all genes from entire genome sequences, cDNA cloning identifies only those genes that are actually expressed. In the present study, a single gene was identified in each species, suggesting that only one gene is expressed in the respective organisms. That is, although two or more paramyosin genes may be present on the genome, some are considered to be pseudogenes. Pseudogenes have base sequences similar to those of normal genes, although they are not expressed *in vivo*. For the species that are considered to have two or more paramyosin genes, the expression of these genes will be further investigated *in vivo*.

5. Conclusions

The primary structures of four squid paramyosins were determined. In each sequence, heptad repeats (*abcdefg*) were identified, as well as hydrophobic amino acids in many of the *a* and *d* positions and charged amino acids in the *e* and *g* positions, representing a characteristic coiled-coil structure. A highly conserved ACD consisting of 29 residues was found in the C-terminal region, through which paramyosin molecules are suggested to polymerize.

The results of phylogenetic analysis showed that the four squid paramyosins form a group independent from the paramyosins of other species, to which octopus paramyosins are most closely related. Fluke and tapeworm paramyosins also formed independent groups, whereas shellfish paramyosins remained dispersed.

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