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Molecular Cloning and Tissue Distribution of Troponin C from the Japanese Pearl Oyster, *Pinctada fucata*

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Abstract

Troponin C (TnC) is one of the subunits of troponin. Troponin, which is activated by Ca^{2+} binding, is a thin filament-associated regulator of vertebrate striated muscle contraction. The function of TnC in vertebrates has been characterized in detail, but the role of TnC in molluscan muscles is still unclear. In this work, we investigated whether TnC plays a role in the catch contraction of molluscan smooth muscle in the bivalve Japanese pearl oyster *Pinctada fucata*. We determined the full-length primary structure of the TnC protein from the *P. fucata* adductor muscle (Pifuc-TnC), and found it is composed of 150 amino acid residues with a predicted molecular weight of 17,400. Multiple sequence alignments indicated that it had four EF-hand motifs, but only one (site IV) was predicted to have Ca^{2+}-binding ability. This is analogous to characterized TnCs from other mollusks. Three-dimensional modeling of Pifuc-TnC using SWISS-MODEL indicated the presence of a short loop within the α-helix connecting the site II and III EF-hand motifs. We predicted the gene structure of Pifuc-TnC using Splign alignment of our obtained cDNA and genome sequences and elucidated that Pifuc-TnC consists of five exons, with the start and stop codons located in exon 1 and exon 5, respectively. Using quantitative real-time PCR, we determined that the Pifuc-TnC gene is predominantly expressed in adductor phasic muscle and rarely in adductor catch muscle, gill, mantle and foot. These findings suggest that TnC may not have a role in catch muscle contraction.

Keywords

Adductor Muscle, Catch Contraction, EF-Hand, Troponin, Troponin C

1. Introduction

Troponin (Tn) is the sarcomeric Ca^{2+}-dependent regulator for striated muscle

contraction in vertebrates. It is distributed on thin filaments and inhibits the interaction between actin and myosin. Troponin consists of three subunits: troponin C (TnC), troponin I (TnI), and troponin T (TnT). The binding of Ca^{2+} to TnC induces a conformational change in the troponin complex structure and enables myosin to interact with actin [1]-[6].

All characterized TnCs consist of four EF-hand motifs, which possess helix-loop-helix topology and are designated as sites I-IV (from the N-terminus). Although all four vertebrate fast skeletal TnC EF-hand motifs are capable of binding Ca^{2+}, only TnC sites II and IV are able to bind Ca^{2+} in arthropod and nematode striated muscles [7] [8]. In mollusks such as scallop and squid, only site IV is able to bind to Ca^{2+} [9] [10]. The ability of an EF-hand motif to bind Ca^{2+} is dependent upon its primary structure [11].

Both vertebrate and molluscan muscle contraction are regulated by intracellular Ca^{2+} concentrations [12]. However, in contrast to vertebrates, mollusks employ a thick filament-linked regulatory system where myosin binds Ca^{2+} directly leading to its activation and subsequent interaction with actin. Although Tn is also located in molluscan muscles, it is currently unclear whether it is involved in a similar thin filament-linked regulatory system to that in vertebrates.

Bivalve adductor muscles are composed of two muscle types: phasic and catch. The large phasic muscle is used for quick closure of shells, whereas the smaller catch muscle is involved in the sustainable closure of shells. Catch muscles can develop a long-lasting high tension state with little energy expenditure. They begin to contract following an increase in intracellular Ca^{2+} concentrations, which activates myosin and develops the tension. They subsequently enter the catch state once Ca^{2+} concentrations decrease to resting levels [12]. In the catch state, thin and thick filaments are thought to be tethered together by a complex of myosin, actin, and twitchin, a giant myosin-associated protein [13] [14] [15]. However, there are currently no data to suggest that thin filament-linked regulation is involved in catch contraction.

The genome database of the pearl oyster *Pinctada fucata* has been completely determined and we have already located and annotated genes encoding fundamental muscle proteins [16] [17] [18]. Recently, it has been reported that gene expression patterns differ between scallop phasic and catch muscles as revealed by proteomic and transcriptomic analyses. Troponin is expressed in phasic muscle higher than catch muscle, indicating a different regulatory system might be employed in each muscle [19]. However, there are little data available to suggest the function of all the elucidated muscle proteins in molluscan muscle contraction. Therefore, in this study, we performed a molecular characterization of *Pinctada fucata* troponin C (Pifuc-TnC) to investigate if it is involved in catch contraction.

2. Materials and Methods

2.1. Pearl Oysters

We obtained live specimens of the Japanese pearl oyster, *Pinctada fucata* that

were cultured in Ago Bay, Mie Prefecture, Japan. The adductor muscle, gill, mantle, and foot were dissected from each oyster body, immediately frozen in liquid nitrogen, and stored at −80˚C until use.

2.2. cDNA Cloning of *Pinctada fucata* Troponin C

Total RNA was extracted from the phasic part of the adductor muscle using a conventional method [20]. First strand cDNA was synthesized using the 3'-Full RACE Core Set (TaKaRa-Bio, Ohtsu, Japan) using the total RNA as the template. Three 3' RACE primers were designed based on the partial sequence of Pifuc-TnC: 5'-GTAGAGGACTTAAGGTGGAT-3' for the first PCR, 5'-TAAAATCGTTAGGTGATGAT-3' for the nested PCR, and 5'-TTCACCATAAAGGTCACCCT-3' for the second nested PCR [18]. PCR was carried out using SapphireAmp Fast PCR Master Mix (TaKaRa-Bio) with the forward primers detailed above and the Oligo dT-3 sites adaptor primer. PCR conditions were as follows: 30 cycles of denaturation at 98˚C for 5 s, annealing at 55˚C for 5 s, and elongation at 72˚C for 20 s. The amplified DNA fragment was sequenced after insertion into a pTAC-1 vector (BioDynamics Laboratory Inc., Tokyo, Japan). 5' RACE was carried out using the 5' RACE system for Rapid Amplification of cDNA Ends, version 2.0 (Invitrogen, Carlsbad, CA, USA). Three primers were designed using the sequence determined by 3' RACE: 5'-CGTCACTCCATTCTTTGAGT-3' for synthesizing cDNA, 5'-CTCATCGTCAACTTGAAGTC-3' for the first PCR, and 5'-AAACTTTTAAAAACTTTTTC-3' for the second PCR. PCR was carried out using SapphireAmp Fast PCR Master Mix with the forward primers detailed above and the primers included in the kit. PCR conditions were as follows: 30 cycles of denaturation at 98˚C for 5 s, annealing at 55˚C for 5 s, and elongation at 72˚C for 10 s. The amplified DNA fragment was sequenced after insertion into a pTAC-1 vector. The determined sequence has been registered in DDBJ/EMBL/GenBank (accession number LC381286).

2.3. Comparison of the Primary Structure of Troponin C with Those of Other Species

The primary structure of Pifuc-TnC was deduced from the nucleotide sequence determined by cDNA cloning and compared with those from mollusks using ClustalW: akazara scallop *Chlamys nipponensis akazara* (BAA12908), asari clam *Ruditapes philippinarum* (AFB83400), and squid *Todarodes pacificus* (Q9BLG0); arthropods: acorn barnacle *Balanus nubilus* (P21798), American lobster *Homarus americanus* (P29289), and fruit fly *Drosophila melanogaster* (NP_476968); nematode: *Caenorhabditis elegans* (BAB84566); vertebrates: chicken *Gallus gallus* (NP_990781), salmon *Salmo salar* (ACH70760), clawed frog *Xenopus laevis* (NP_001079408), rabbit *Oryctolagus cuniculus* (NP_001076114), and human *Homo sapiens* (NP_003270).

2.4. Three-Dimensional (3D) Modeling of Troponin C Structures

The 3D structure of Pifuc-TnC was predicted with SWISS-MODEL [21] using

the PDB data of chicken TnC (PDB: 1YTZ) as a template. To compare the structures of different TnCs, American lobster TnC (NCBI sequence FJ790224) was also modeled following the same method.

2.5. Gene Structure of *P. fucata* Troponin C

The genome sequence including the Pifuc-TnC gene was obtained by BLAST searching the Pifuc-TnC nucleotide sequence against the *P. fucata* genome database [17]. The gene structure of the Pifuc-TnC gene was predicted by analyzing cDNA and genome sequences using the Splign alignment tool (NCBI) [22].

2.6. Gene Expression Analysis of *P. fucata* Troponin C in Tissues

Gene expression patterns of Pifuc-TnC in the catch and phasic muscles, gill, mantle and foot were analyzed by quantitative real-time PCR. cDNAs were synthesized using total RNA from each tissue as templates using RiverTra Ace® qPCR RT Master Mix (Toyobo Co., Ltd., Osaka, Japan). Primers and a probe were designed by Universal Probe Library Assay Design Center (Roche Diagnostics, Mannheim, Germany) using the full-length nucleotide sequence determined in this study. The primers used were: 5'-TTAACAGACGAAGAACTCGATGA-3' (forward) and 5'-TGTCCCTGAGCCGTCTGT-3' (reverse). Probe #94 (Roche Diagnostics) was used as a TaqMan probe. *P. fucata* β-actin (AF378128) was used as an internal standard. The primers used for β-actin were as follows: 5'-TCGTTCCTCGGAATGGAA-3' (forward), 5'-TCGACATCGCATTTGAGAAT-3' (reverse). Probe #151 (Roche Diagnostics) was used as a TaqMan probe. The PCR reaction was performed using Eagle Taq Master Mix with ROX (Roche Diagnostics).

3. Results

3.1. Molecular Characteristics of *P. fucata* Troponin C

The full-length nucleotide sequence of Pifuc-TnC was obtained through cDNA cloning and was found to contain 1775 nucleotides (nt), which includes an open reading frame of 453 nt, a 77 nt 5' untranslated region, and a 1245 nt 3' untranslated region (**Figure 1**). It encodes a protein of 150 amino acid residues in length with a predicted molecular weight of 17,400 (**Figure 1**).

The amino acid sequence of Pifuc-TnC was 68% homologous to both asari clam and squid TnCs and was 64% homologous to akazara scallop TnC. In contrast, the Pifuc-TnC amino acid sequence exhibited between 30 and 40% homology to TnC from arthropods, nematodes and vertebrates (**Table 1**).

Multiple sequence alignments of Pifuc-TnC with TnCs from other organisms revealed that Pifuc-TnC has four potential EF-hand motifs, termed sites I, II, III and IV. The Ca^{2+} binding capability of an EF-hand motif can be predicted through analysis of its amino acid sequence [11]. Site I in Pifuc-TnC is predicted to have no Ca^{2+} binding ability as the third residue, which is required to be aspartic acid, asparagine or serine in order to bind to Ca^{2+}, is a lysine. In site II,

```
GACACACGGATTATAGGGAAAATATAGGTGTCCAGCTTTGCTTAGATTCTTTGCTTTCGG    60
ATTGGTTTCTCTATCGTCATGTCGGAATTTAAAGTATCGGAAAAACAATTTGCAGATGCC   120
                     M  S  E  F  K  V  S  E  K  Q  F  A  D  A     14
CATCAGACGTTTAACCTGTTTGACAAGAAGGGTACAGGCCAAGTGTCCACTAAAGAGTTA   180
 H  Q  T  F  N  L  F  D  K  K  G  T  G  Q  V  S  T  K  E  L     34
GAAAAAGTTTTTAAAAGTTTAGCACTTCAAGTTGACGATGAGAAACTCAAAGAATGGAGT   240
 E  K  V  F  K  S  L  A  L  Q  V  D  D  E  K  L  K  E  W  S     54
GACGAGCTTGATGAAGAGGCTACCGGATATTTCACATGGGATCAATTTAAGATCCTTTTT   300
 D  E  L  D  E  E  A  T  G  Y  F  T  W  D  Q  F  K  I  L  F     74
GAGAGGAAGTTGAAAACGGACGAAGATGAGAGAGAATTACGGGAAGCATTCAGAGTGTTA   360
 E  R  K  L  K  T  D  E  D  E  R  E  L  R  E  A  F  R  V  L     94
GACAAGGGAAATAAAGGCACAATTCCAGTAGAGGACTTAAGGTGGATTTTAAAATCGTTA   420
 D  K  G  N  K  G  T  I  P  V  E  D  L  R  W  I  L  K  S  L    114
GGTGATGATTTAACAGACGAAGAACTCGATGAAATGATAGCGGAGACAGACACAGACGGC   480
 G  D  D  L  T  D  E  E  L  D  E  M  I  A  E  T  D  T  D  G    134
TCAGGGACAGTAGATTATGAAGAATTCAAAACGCTGATGACGTCAGACTGATGAACTCAT   540
 S  G  T  V  D  Y  E  E  F  K  T  L  M  T  S  D  *             150
ATCGAGGCTGGACACCGGCCACATCCCTCTCTGTACGCACGCGTCATTCATCAGATTGCT   600
GTTAAGTTTGTAATACGATGGACTCTGTTCCCTGCTCCAGACAAAGTTTGGATGTATATT   660
CTTTGCGGCGTACTTCGTCCTCGGCGATATTTATCTGCAGTTGTAGTTTCCAACGATAAT   720
ATACGTTCAAAAACTTGTTATCTTCTTTTGAAATTTTGAATTGATTGCTTCCCATCATTC   780
CATTTAACACATGCGCACTAATTGCTCTAACCCCAGAATGCATTAGCTTTGCTTGCTGCT   840
TTGCACCAACTTGCTGTCTTCCCCTATCGCGCAGCTCCACAGCACTTTTTTCTTCTCTTA   900
TGCTATGTTTTATATATTGTTTTCAGAGTTCTTTAAACTGATGTGGGTGATTAAGCGGAC   960
TAGGACGCCCAACTTGCCCGTCTTTGTGGATGTTTGACATGCCCTCCTGTGTGCTTTCAC  1020
CATAAAGGTCACCCTCCTTACCAATCTTACCCCGGAGCTGATTTTTGTATATTTTCATGT  1080
GATATAACTTATTTGTAAATATTCATTTTTTCACATGTTATCCCAACTGATATGTTTTCT  1140
TTTTACATGGAAGGCATATATAGACTGTGTTCATCGTGTTAAGTTCTGTATCTTGCCGAC  1200
ATATTGCACTATCTGCCTGTCTCTATAACATTGGCTTCACTTACTCTGAACTGTTCATGT  1260
CATCGCCAAAAAGTTTAACATTAATTCAGTATATCGCACTATGAATGTGAACTAAACTGA  1320
CAGACTGAGTCATGCTGACTTACAACATGTTATATCTATTTATATTTACATGAGCATGCC  1380
AGATATTTTGAAATGGTACTGAGAGCATATCTATATATCAATTTGATGCAGATTTCATTA  1440
CTAATTCTGTTTTTGACTTTACTCTTTAACTTGCCCAGCTAATATGTGTAAATCATAGAT  1500
AAGTATTTATATATATTCTCCACTTTGTGAATCAAGGACATTATGTATGCATGTGCTGAC  1560
TCCACATCTATGCATTCTCCTTGCCATATCTAGAAAAAAATTATTGTTTTATGTATAATT  1620
GTAATGATTCTGAGAGGAAAATGATTATAATTTGTACATTTGTGAATTTGACTGTAAAGG  1680
GTGTTTTGATGCCCAGAATAAAAGATCCTGTTGCAGCAAATGTATATGAATAAAACAGAA  1740
TATTGAACTTTTCTAAAAAAAAGAAAAAAAAAA                              1775
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Figure 1. Nucleotide and deduced amino acid sequences of *Pinctada fucata* troponin C. The amino acid sequences of the four predicted EF-hand motifs are underlined. The EF-hand motif that is predicted to bind Ca^{2+} by comparing with those from other species is shaded. The numbers on the right of the sequences denote nucleotides from the 5'-end and amino acid residues from the N-terminus. The stop codon is indicated by an asterisk.

Table 1. Percentage identity of the elucidated *Pinctada fucata* troponin C amino acid sequence to troponin C proteins from other species. The predicted Ca^{2+} binding sites (EF-hand motifs) are also indicated.

Phylum	Species	Identity (%)	Ca^{2+} binding site
Mollusca	Pearl oyster *Pinctada fucata*	-	IV
	Akazara scallop *Chlamys nipponensis akazara*	64	
	Asari clam *Ruditapes philippinarum*	68	
	Squid *Todarodes pacificus*	68	
Arthropoda	Acorn barnacle *Balanus nubilus*	35	II, IV
	American lobster *Homarus americanus*	32	
	Fruit fly *Drosophila melanogaster*	39	
Nematoda	Nematode *Caenorhabditis elegans*	38	
Chordata	Chicken *Gallus gallus*	30	I, II, III, IV
	Salmon *Salmo salar*	33	
	Xenopus *Xenopus laevis*	34	
	Rabbit *Oryctolagus cuniculus* fast skeletal	31	
	Human *Homo sapiens* fast skeletal	31	

glutamic acid, phenylalanine and glutamine were identified as the third, eighth and twelfth residues, respectively, and in site III, glycine, lysine and proline were identified as the third, fifth and ninth residues, respectively. Therefore, sites II and III are also predicted to be unable to bind to Ca^{2+}. Only site IV satisfied the amino acid requirements for Ca^{2+} binding, which is analogous to known TnCs from other mollusks (Figure 2 and Table 1). Therefore, out of the four potential

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                                                 Site I
Pearl oyster      ------MSEFKVSEK----QFADAHQTFNLFDKKGTGQVSTKELEKVFKSLALQVDDEKL  50
Akazara scallop   -----MSDEFRATEK----QISDAKQAFCNFDKKKEGTVSCKDLGAIFKSLGLVMKDDKI  51
Asari clam        ------MTDFKVTDK----QFTDAKSTFHLYVKKGSEEVATKDLDQLFKAMALHIDDEKL  50
Squid             -------MPVVISEK----QFNDAHQAFKLHDKKDEGAVSNKELTNLFKSLALHVSDDKL  49
Acorn barnacle    -------MMDELDKD----QIAMLKKAFDGFDHEKKGAINCDVVATILRMMGQAYNAQTL  49
American lobster --------MDTLDED----QVQALQKAFNSFDTDDKGFITPDTVGVILRMMGVKISDRHL  48
Fruit fly         --------MDNIDEDLTPEQIAVLQKAFNSFDHQKTGSIPTEMVADILRLMGQPFDRQIL  52
Nematode          MGDVVADALEKLSAD----QIEQFRKYFNMFDKEGKGYIRATQVGQILRTMGQAFEERDL  56
Chicken           -MASMTDQQAEARAFLSEEMIAEFKAAFDMFDADGGGDISTKELGTVMRMLGQNPTKEEL  59
Salmon            ----MTDAQQEARSFLSEEMLNEFKAAFDMFDTDGGGDISTKELGQVMRMLGQNPTRQEL  56
Clawed frog       -MAQPTDQQQDARSFLSEEMIAEFKAAFDMFDTDGGGDISTKELGTVMRMLGQTPTKEEL  59
Rabbit            ----MTDQQAEARSYLSEEMIAEFKAAFDMFDADGGGDISVKELGTVMRMLGQTPTKEEL  56
Human             ----MTDQQAEARSYLSEEMIAEFKAAFDMFDADGGGDISVKELGTVMRMLGQTPTKEEL  56
                                Site II                                Site III
Pearl oyster      KEWSDELDEEATGYFTWDQFKILFERKLKT-DEDE---RELREAFRVLDKGNKGTIPVED 106
Akazara scallop   KDWSDEVDEEATGRLSCEQWLKLFEWKLKE-DLDE---RELKEAFRVLDKEKKGVIKVDV 107
Asari clam        KDWADEMDEDATGLITWEKFKVLFERKLKE-DEEE---KELKEAFRVLDSQKKGVIPVSD 106
Squid             QQWVDEMDEDATGVIRWEKFKILFERKVQE-DEDE---RELRSAFRVLDKNNQGVIDVED 105
Acorn barnacle    KELIDEVDADGSGMLEFEEFVTLAAKFIID-DDAEAMAKELKEAFRLYDKAGKGYIPTSA 108
American lobster  QEVISETDEDGSGEIEFEEFAALAAKFLSE-EDEEALKKELKEAFRIYDRGGNGYITVHT 107
Fruit fly         DELIDEVDEDKSGRLEFEEFVQLAAKFIVE-EDDEAMQKELREAFRLYDKQGNGYIPTSC 111
Nematode          KQLIKEFDADGSGEIEFEEFAAMVANFVVNNENDEGLEEELREAFRLYDKEGNGYINVSD 116
Chicken           DAIIEEVDEDGSGTIDFEEFLVMMVRQMKE-DAKGKSEEELANCFRIFDKNADGFIDIEE 118
Salmon            DEIIEEVDEDGSGTIDFEEFLVMMVRLLKE-DQAGKSEEELAECFRVFDKNADGYIDREE 115
Clawed frog       DAIIEEVDEDGSGTIDFEEFLVMMVRQMKE-DAQGKSEEELAECFRIFDKNADGYIDGEE 118
Rabbit            DAIIEEVDEDGSGTIDFEEFLVMMVRQMKE-DAKGKSEEELAECFRIFDRNADGYIDAEE 115
Human             DAIIEEVDEDGSGTIDFEEFLVMMVRQMKE-DAKGKSEEELAECFRIFDRNADGYIDPEE 115
                                          Site IV
Pearl oyster      LRWILKSLGDDLTDEELDEMIAETDTDGSGTVDYEEFKTLMTSD-- 150
Akazara scallop   LRWILKSLGDELTEDEIENMIAETDTDGSGTVDYEEFKCLMMSSDA 153
Asari clam        LRWILKSLGDDITEEEIDDMIAETDTDGSGTVDYEEFKSLMSSE-- 150
Squid             LRWILKSLGDDLNDDEIQDMINETDTDGSGTVDYEEFSALMLG--- 148
Acorn barnacle    LKDILKELDETLNAEDLDNIIGEIDTDGSGTVDFDEFMEMMTG--- 151
American lobster  LKEILRELDNKLTEDNLDSIIEEVDEDGSGTIDFNEFMKMMNG--- 150
Fruit fly         LKEILKELDDQLTEQELDIMIEEIDSDGSGTVDFDEFMEMMTGE-- 155
Nematode          LRDILRALDDNVSEEELDEMIAEIDADGSGTVDFDEFMEMMSGE-- 160
Chicken           LGEILRATGEHVIEEDIEDLMKDSDKNNDGRIDFDEFLKMMEGVQ- 163
Salmon            FAIIIRSTGEQISEEEIDELLKDGDKNADGMLDFDEFLKMMENVQ- 160
Clawed frog       LAEILRSSGESITDEEIEELMKDGDKNNDGKIDFDEFLKMMEGVQ- 163
Rabbit            LAEIFRASGEHVTDEEIESLMKDGDKNNDGRIDFDEFLKMMEGVQ- 160
Human             LAEIFRASGEHVTDEEIESLMKDGDKNNDGRIDFDEFLKMMEGVQ- 160
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Figure 2. Multiple sequence alignments of the deduced sequence of *Pinctada fucata* troponin C with those from various species. The putative EF-hand motifs are highlighted in yellow (predicted to bind to Ca^{2+}) and gray (predicted to be unable to bind to Ca^{2+}). Compared sequences were obtained from the NCBI database: akazara scallop *Chlamys nipponensis akazara* (BAA12908), asari clam *Ruditapes philippinarum* (AFB83400), squid *Todarodes pacificus* (Q9BLG0), acorn barnacle *Balanus nubilus* (P21798), American lobster *Homarus americanus* (P29289), fruit fly *Drosophila melanogaster* (NP_476968), nematode *Caenorhabditis elegans* (BAB84566), chicken *Gallus gallus* (NP_990781), salmon *Salmo salar* (ACH70760), clawed frog *Xenopus laevis* (NP_001079408), rabbit *Oryctolagus cuniculus* (NP_001076114), and human *Homo sapiens* (NP_003270). Numbers on the right represent the numbers of the amino acid residues from the N-terminus. Gaps were inserted to optimize the sequence alignments.

Ca^{2+} binding sites in Pifuc-TnC, site IV is the only EF-hand motif that is predicted to bind to Ca^{2+}. To confirm our prediction, we are planning to make a recombinant Pifuc-TnC and its variants to be subjected to Ca^{2+}-binding assays. A 3D model of Pifuc-TnC predicted using SWISS-MODEL was very similar to TnCs from chicken fast skeletal muscle and American lobster. The only notable difference was the presence of a short loop (four amino acids) within the *α*-helix connecting the site II and III EF-hand motifs (**Figure 3**). Similar structures were also predicted for TnC from other mollusks: akazara scallop and squid (data not shown). This structural divergence does suggest functional differences between molluscan and vertebrate TnCs. Ca^{2+} binding by vertebrate fast skeletal TnC involves all four EF-hand motifs and leads to drastic conformational changes to trigger the interaction between myosin and actin. Although it has been reported that the conformation of molluscan TnC does change upon Ca^{2+} binding, the degree of the structural change and if it consequently modifies the role of troponin in the regulation of molluscan muscle contraction remain unknown [23]. Further studies are required to unveil the role of troponin in molluscan muscle.

3.2. Gene Structure of the *P. fucata* Troponin C Gene

BLAST searching of our obtained Pifuc-TnC nucleotide sequence against the genome database of *P. fucata* yielded a single nucleotide sequence at scaffold 1306.1. In our previous study, we annotated a gene model (pfu_aug1.0_1306.1_22530) that was automatically predicted by the genome database to be TnC [18]. The gene model contained the predicted full-length amino acid sequence of TnC, which is identical to the sequence determined in this study. We then predicted the gene structure of Pifuc-TnC using Splign alignment of the obtained cDNA and genome sequences. Pifuc-TnC consists of five exons (**Figure 4**), and the start and stop codons are located in exon 1 and exon 5, respectively.

3.3. Distribution of Troponin C in *P. fucata* Tissues

Pifuc-TnC was predominantly expressed in phasic adductor muscle, while weak expression was detected in catch adductor muscle, gill, mantle and foot (**Figure 5**). TnC is a key regulator in the fast contraction of vertebrate striated muscles (skeletal and cardiac muscles). The phasic adductor muscle is thought to control the quick closure of shells. Our findings suggest that TnC could be involved in the regulation of the phasic adductor muscle, rather than the catch adductor muscle. However, it has been reported that TnC was isolated from both the phasic and catch adductor muscles of the akazara scallop [24]. It is possible that the function of TnC is species-dependent.

4. Discussion

In this study, we have analyzed the molecular characteristics of Pifuc-TnC. Our findings have indicated that Pifuc-TnC has a similar structure to known molluscan TnCs, indicating that they could play analogous roles in muscle contraction.

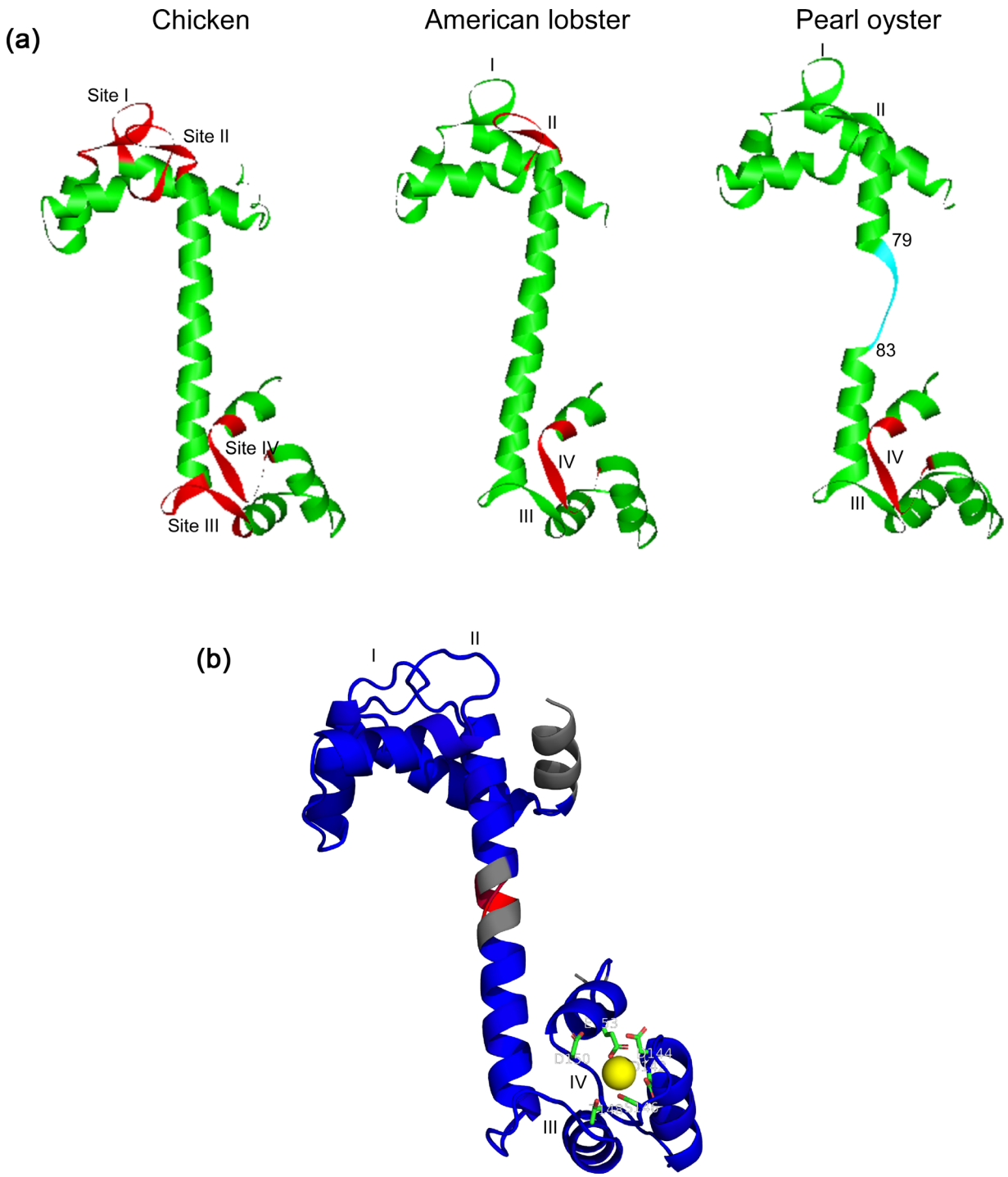

Figure 3. Three-dimensional modeling of troponin C. (a) Three-dimensional models of troponin C from chicken fast skeletal muscle, American lobster and pearl oyster. Troponin C models of American lobster and pearl oyster were predicted using the SWISS-MODEL program using the PDB data from chicken troponin C (PDB: 1YTZ) as a template. The amino acid sequence of American lobster troponin C was obtained from the NCBI database (FJ790224). EF-hand motifs predicted to bind to Ca^{2+} are shown in red. The region that does not form an *α*-helix structure in the pearl oyster troponin C model is shown in blue. The numbers in the pearl oyster troponin C model represent numbers of amino acid residues from the N-terminus. (b) The superimposed image of the three-dimensional models from chicken fast skeletal and pearl oyster troponin Cs. The image is colored by RMSD. Side chains of amino acid residues considered to be involved in Ca^{2+} binding are represented by sticks. The Ca^{2+} atom is indicated in yellow.

Figure 4. Gene structure of *Pinctada fucata* troponin C. Black boxes indicate exons, and intervening lines represent introns. The ATG start codon and TGA stop codons are indicated.

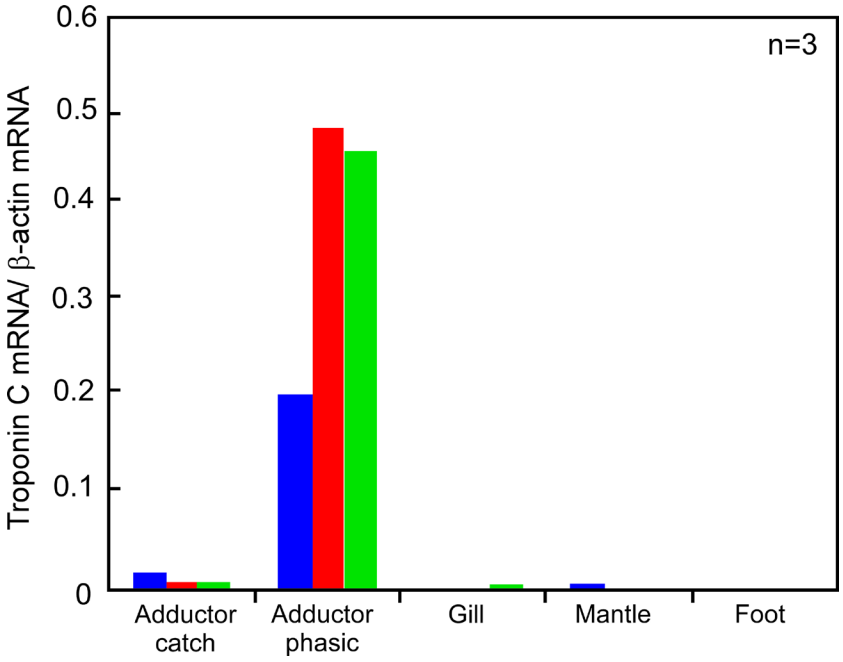

Figure 5. Gene expression patterns of troponin C in *Pinctada fucata* tissues. Quantitative real-time PCR analysis was performed to examine troponin C gene expression in *Pinctada fucata* adductor catch muscle, adductor phasic muscle, gill, mantle, and foot. The data shown are from three independent experiments. The y-axis indicates relative troponin C expression levels using *β*-actin as an internal standard.

Pifuc-TnC and other characterized molluscan TnCs have four EF-hand motifs, but only one is predicted to bind to Ca^{2+}. In contrast, all four vertebrate fast skeletal TnC EF-hand motifs are able to bind Ca^{2+}. The predicted 3D models of molluscan TnCs also differ substantially from those of vertebrate fast skeletal TnCs. To date, numerous studies have accumulated a large amount of data on vertebrate TnC function. However, the observed divergences in structure and Ca^{2+} binding mean it is impossible to extrapolate these findings to the function of molluscan TnCs. Indeed, it has been reported that akazara scallop TnC is likely to function in a different manner to vertebrate TnCs [23] [25].

The Pifuc-TnC gene is predominantly expressed in phasic muscle and not in catch muscle, suggesting that in the pearl oyster *P. fucata*, TnC may be involved in the regulation of phasic muscle contraction. We have previously reported that isoforms of twitchin, a known regulator of catch muscle contraction, are expressed in both phasic and catch muscles, and indicated that the divergent properties of these muscle types might be attributed to the presence of different

twitchin isoforms [26]. It is also possible that troponin is able to preclude twitchin-regulated catch contraction activity in phasic muscle.

Troponin is a complex of three subunits: TnC, together with TnI and TnT. Our previous studies have elucidated partial sequences of Pifuc-TnI and Pifuc-TnT genes in the genome database [18]. Further molecular characterization studies on Pifuc-TnI and Pifuc-TnT are required to clarify the function of troponin in *Pinctada fucata*.

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