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

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Abstract

Troponin is a complex of three proteins (troponin I, troponin C, and troponin T) that binds Ca^{2+} and is a thin filament-associated regulator of vertebrate striated muscle contraction. The function of troponin I (TnI) in vertebrates has been extensively characterized, but its role in molluscan muscles has not yet been elucidated. Our previous work suggested that the troponin C subunit has a role in adductor phasic muscle but not in catch muscle. Here, we investigated the molecular characteristics of TnI from the bivalve Japanese pearl oyster, *Pinctada fucata* to aid the elucidation of the function of molluscan muscle troponin. We determined the primary structure of the full-length TnI protein from the *P. fucata* adductor muscle (Pifuc-TnI) and found that it is composed of 286 amino acid residues with a predicted molecular weight of 33,737. Motif structure predictions and multiple sequence alignments revealed that Pifuc-TnI has a 138 residue extension at its N-terminus compared with rabbit TnI. This is analogous to characterized TnIs from other mollusks. However, unlike scallop TnI, Pifuc-TnI is predicted to contain two cAMP-dependent protein kinase phosphorylation sites, at residues 39 - 45 (RRGTEDD) and 145 - 151 (KKKSKRK). Phylogenetic analysis indicated that Pifuc-TnI and molluscan TnIs were grouped into the same clade. *Pifuc-TnI* gene structure predictions using Splign alignment of our obtained cDNA and genome sequences indicated that *Pifuc-TnI* consists of fifteen exons, with the start and stop codons located in exon 2 and exon 11, respectively. Using quantitative real-time PCR, we determined that the *Pifuc-TnI* gene is predominantly expressed in adductor phasic muscle, weakly in adductor catch muscle, and is not expressed in the gill, mantle or foot. These findings suggest that TnI, as a component of the troponin complex, plays a regulatory role in adductor phasic muscle contraction, but not in catch contraction.

Keywords

Adductor Muscle, Catch Contraction, *Pinctada fucata*, Troponin, Troponin I

1. Introduction

Mollusk bivalve adductor muscles are composed of two muscle types: phasic and catch. Phasic muscle is used for the quick closure of shells, whereas catch muscle is involved in the sustainable closure of shells. The contraction of both muscles is regulated by intracellular Ca^{2+} concentrations [1]. Mollusks employ a thick filament-linked regulatory system where myosin directly binds Ca^{2+}, leading to its activation and subsequent interaction with actin. Following a decrease in the intracellular Ca^{2+} concentration, myosin is inactivated, and its interaction with actin in phasic muscle is abolished. Once Ca^{2+} concentrations decrease to resting levels, catch muscle enters the high-tension catch state, which is able to be maintained for long periods of time. Twitchin, a giant myosin-associated protein, is thought to tether together the thin and thick filaments through its phosphorylation sites [2] [3] [4].

In contrast to molluscan muscles, vertebrate striated muscles employ a thin filament-linked regulatory system. Troponin (Tn) is the regulator of skeletal muscle contraction. It is distributed on thin filaments and inhibits the interaction between actin and myosin. Tn 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 [5] [6] [7] [8]. 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.

The Japanese pearl oyster, *Pinctada fucata*, is one of the most important molluscan species in the pearl culture industry. A genome database of *Pinctada fucata* has recently been released and all the major muscle protein genes have been registered [9] [10] [11]. Therefore, we have used *P. fucata* as a model system to elucidate the molluscan muscle regulatory system. Our studies have focused on the adductor muscle Tn subunits to investigate the molecular mechanism of thin filament-linked regulation of molluscan muscle contraction. To this end, we recently characterized *P. fucata* TnC (Pifuc-TnC) [12] [13]. Pifuc-TnC has four EF-hand motifs, sites I-IV, and, like other molluscan TnCs [14] [15] [16], only site IV is able to bind Ca^{2+} [12]. A conformational change is induced in Pifuc-TnC upon Ca^{2+}-binding [12]. A predicted three-dimensional model of Pifuc-TnC closely resembled that of vertebrate TnC, apart from a short loop (four amino acids) in the former structure that breaks the α-helix connecting the N- and C-terminal lobes [13]. Quantitative real-time PCR indicated that the *Pifuc-TnC* gene is predominantly expressed in adductor phasic muscle, suggesting a role for Tn in adductor phasic muscle regulation [13]. Furthermore, recent

proteomic and transcriptomic analyses have revealed that scallop phasic and catch muscles have different gene expression patterns [17]. Together, these findings suggest that adductor phasic and catch muscles employ different regulatory systems.

The interaction between TnC and TnI in the vertebrate troponin complex is dependent upon Ca^{2+} binding to TnC [18] [19] [20] [21]. Characterized TnIs from other scallop species have been found to have a long (>130 residues) N-terminal extending region that is absent in vertebrate striated muscle TnI [22] [23]. A binding analysis study using recombinant scallop TnI domains revealed that an N-terminal peptide (residues 130 - 252) that lacks the extension but contains a region homologous to residues 1 - 30 of rabbit TnI is able to bind to both TnC and actin [23]. However, little is known about molluscan TnIs and further studies are required to unveil the function of Tn in the regulation of molluscan muscle contraction.

Our research is focused on the elucidation of Tn function in molluscan muscle contraction. To perform a molecular characterization of *P. fucata* TnI (Pifuc-TnI), we analyzed the full nucleotide sequence, the gene structure, and *Pifuc-TnI* tissue expression patterns.

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 I

Total RNA was extracted from the phasic part of the *Pinctada fucata* adductor muscle using a conventional method [24]. The partial nucleotide sequences of the *P. fucata* troponin I (*Pifuc-TnI*), as determined by 3′rapid amplification of cDNA ends (RACE), have been previously reported [10]. To determine the full-length sequence of *Pifuc-TnI*, 5′RACE was carried out using the 5′RACE system for Rapid Amplification of cDNA Ends, version 2.0 (Invitrogen, Carlsbad, CA, USA) using total RNA as a template. Primers were designed using the elucidated sequences of *Pifuc-TnI*: 5′-GCCATATGAGCTCACTGAAGGT-3′ for synthesizing cDNA, 5′-TCCTCGAGTTATCTACTATGTT-3′ for the first PCR, and 5′-AAGAATAGCTCGAGGATCCGG-3′for the second PCR. PCR was carried out using SapphireAmp Fast PCR Master Mix (TaKaRa Bio, Otsu, Japan) with the forward primers detailed above and the reverse primers included in the kit. The 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 nucleotide sequence data were registered in the DDBJ/EMBL/GenBank sequence database (accession number LC458449).

2.3. Comparison of the Primary Structure of *P. fucata* Troponin I with Those from Other Species

The Pifuc-TnI primary structure was determined from the *Pifuc-TnI* cDNA nucleotide sequence. ClustalW (https://www.ddbj.nig.ac.jp/index-e.html) was used to compare this sequence with Troponin I amino acid sequences from other molluscan species: Japanese scallop, *Chlamys nipponensis* (AB206837); Yesso scallop, *Mizuhopecten yessoensis* (AB008005, AB008006); and rabbit, *Oryctolagus cuniculus* (S61403).

2.4. Prediction of Structural Motifs in *P. fucata* Troponin I

The motif structure of Pifuc-TnI was predicted by SMART (http://smart.embl-heidelberg.de/), Pfam (http://pfam.xfam.org/), and the Eukaryotic Linear Motif resource (http://elm.eu.org/).

2.5. Phylogenetic Analysis of Troponin I

Phylogenetic analysis was carried out using the primary structures of troponin I from various species following sequence alignment using the ClustalW algorithm. The sequences used were: human, *Homo sapiens* (AAA19813.1); mouse, *Mus musculus* (AAA40485.1); rabbit, *Oryctolagus cuniculus* (AAB26824.1); rat, *Rattus norvegicus* (AAA42294.1); pig, *Sus scrofa* (AAP37479.1); common quail, *Coturnix coturnix* (AAB00122.1); chicken, *Gallus gallus* (AAA61952.1, NP_998735.1); tropical clawed frog, *Xenopus tropicalis* (AAH61268.1, AAH84508.1); African clawed frog, *Xenopus laevis* (AAA65727.1, AAL86906.1, NP_001081378.1, NP_001079556.1, NP_001079781.1); Atlantic herring, *Clupea harengus* (AAB05825.1); Orange-spotted grouper, *Epinephelus coioides* (ADG29132.1); rainbow trout, *Oncorhynchus mykiss* (NP_001123462.1); Atlantic salmon, *Salmo salar* (NP_001117133.1); mandarin fish, *Siniperca chuatsi* (ACM07327.1); honey bee, *Apis mellifera* (NP_001035346.1); domestic silkworm, *Bombyx mori* (NP_001037295.1); fruit fly, *Drosophila melanogaster* (CAA42020.1); beet webworm, *Loxostege sticticalis* (ABY56688.1); Japanese scallop, *Chlamys nipponensis* (BAE43657.1, BAE43658.1); akazara scallop, *Chlamys nipponensis akazara* (BAA23775.1) and Yesso scallop, *Mizuhopecten yessoensis* (BAA22852.1, BAA22853.1).

2.6. Gene Structure of *P. fucata* Troponin I

The genome sequence including the *Pifuc-TnI* gene was obtained by BLAST searching the *Pifuc-TnI* nucleotide sequence against the *P. fucata* genome database [9]. The gene structure of the *Pifuc-TnI* gene was predicted by analyzing cDNA and genome sequences using the Splign alignment tool (NCBI) [25].

2.7. Gene Expression Analysis of *P. fucata* Troponin I in Tissues

Gene expression patterns of *Pifuc-TnI* 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). The primers and a probe were designed by the 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′-AGCTGAAGAGGAAACCTCCAC-3′(forward) and
5′-TGGCTATATCGTCCTCAGTGC-3′ (reverse). Probe #18 (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:
5′-TCGTTCCTCGGAATGGAA-3′(forward) and
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 I

The full-length nucleotide sequence of *Pifuc-TnI* was obtained through cDNA cloning and was found to contain 3169 nucleotides (nt), which includes an open reading frame of 861 nt, a 432 nt 5′untranslated region, and a 1936 nt 3′untranslated region (**Figure 1**). It encodes a protein of 286 amino acid residues in length with a predicted molecular weight of 33,737 (**Figure 1**). The amino acid sequence of Pifuc-TnI was 50% homologous to both Japanese scallop and Yesso scallop TnIs and was 21% homologous to rabbit TnI (**Figure 2**). Analogous to known scallop TnIs, Pifuc-TnI has an N-terminal sequence extension of 136 residues compared with rabbit skeletal TnI [22] [23]. This extension is rich in the charged amino acids, glutamic acid and arginine. Motif structure prediction software indicated that Pifuc-TnI contains two coiled-coils (at residues 4 - 25 and 60 - 152) and a troponin motif (residues 161 - 286) (**Figure 3**). In contrast to scallop TnIs, Pifuc-TnI also contains consensus sequences for cAMP-dependent protein kinases (PKAs), which are located at residues 39 - 45 (RRGTEDD) and 145 - 151 (KKKSKRK) (**Figure 2** and **Figure 3**).

BLAST searching of *Pifuc-TnI* against the *P. fucata* genome database yielded two gene models (pfu_aug2.0_433.1_10766.t1 and pfu_aug2.0_433.1_10766.t2) that were computationally predicted from the genome sequence (data not shown). However, neither were found to exactly match our obtained Pifuc-TnI sequence. This suggests the possibility that some TnI isoforms are expressed from a single gene by alternative RNA processing; however, our investigations did not identify any Pifuc-TnI isoform encoding sequences.

Phylogenetic analysis indicated that Pifuc-TnI and molluscan TnIs were grouped into the same clade (**Figure 4**).

3.2. Gene Structure of *P. fucata* Troponin I

BLAST searching of our obtained *Pifuc-TnI* nucleotide sequence against the genome database of *P. fucata* yielded a single nucleotide sequence at scaffold 433.1.

Figure 1. Nucleotide and deduced amino acid sequences of *Pinctada fucata* troponin I. The amino acid sequence is represented in italics. 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.

We then predicted the gene structure of *Pifuc-TnI* using Splign alignment of the obtained cDNA and genome sequences. *Pifuc-TnI* consists of fifteen exons (**Figure 5**), and the start and stop codons are located in exon 2 and exon 11, respectively.

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Pifuc-TnI            MSSLEERREARRRRQQQEEAESSGVAAEEETSTRRSRRRGTEDDIAT--  48
Japanese scallop     ........A..AA..RK.D.N.--.GEEQ...TS......QQ..EE----  44
Yesso scallop        ........A..AA..RK.DDN.--.GEEQ...TS......QE..EE----  44
Yesso scallop 2      ........A..AA..RK.DDN.--.GEEQ...TS......QE..EED.TS  48

Pifuc-TnI            ----------EETDSAPAAVDSTDNAEAERAAQEAAEAEERRRQEEAAER  88
Japanese scallop     -------------..YS.PAEPAYD....NRRRQQQ.E..AAARAAEE.Y  81
Yesso scallop        -------------..YS.PAEPAYD...DNRRRQQQ.E..AAARAAEE.Y  81
Yesso scallop 2      SYRSRRNRGGNDE..YS.PAEPAYD...DNRRRQQQ.E..AAARAAEE.Y  98

Pifuc-TnI            RRREEEEDNRRAEEEERRREEAERRRREEEERAERERLEKEEAEREAARM 138
Japanese scallop     N.QQ..LRRQ.Q...RQ....EQ..QQ.....LRL..E.Q.RE.-..R.. 130
Yesso scallop        N.QQ..LRRQ.Q...RQ....EQ..QQ.....LRL..E.Q.RE.-..R.. 130
Yesso scallop 2      N.QQ..LRRQ.Q...RQ....EQ..QQ.....LRL..E.Q.RE.-..R.. 147
Rabbit Fast Skeletal                                                .   1

Pifuc-TnI            AEEQARKKKSKRKGLGGLDPEKKKKLKMFIMCRAKDELVREAKEKALEKE 188
Japanese scallop     ....KK...---.....S.....M..KL..QK.AED.AN...A..EA.. 177
Yesso scallop        ....KK...---.....S.....M..KL..QK.AED.KN...A..EA.. 177
Yesso scallop 2      ....KK...---.....S.....M..KL..QK.AED.KN...A..EA.. 194
Rabbit Fast Skeletal GD.EK.NRA---------ITARRQH..SVMLQI.AT..EK.EGRREA..Q  42

Pifuc-TnI            NYINSKMPNCQTDSLSEAELIKLCKSLHDVVAKLEEELYDTEQKIRKQDY 238
Japanese scallop     K...DLV.KFS..GKDV.A.QA...DF.KRL.S...DV..W.A.....F 227
Yesso scallop        K...DLV.KFS..GKDV.A.QA...DF.KKL.S...DV..W.A.....F 227
Yesso scallop 2      K...DLV.KFS..GKDV.A.QA...DF.KKL.S...DV..W.A.....F 244
Rabbit Fast Skeletal ..LAEHC.PLSLPG-.M..VQE...Q..AKIDAA...K..M.I.VQ.SSK  91

Pifuc-TnI            DINALTLKINDAKGKFIKPVLRKVNK-ESKFDK--LAKS---KADFRENL 282
Japanese scallop     E..E....V..T....V.........T...L..--IQRKEAK.S...D.. 275
Yesso scallop        E..E....V..T....V.........T...L..--IQRKEAK.S...D.. 275
Yesso scallop 2      E..E....V..T....V.........T...L..--IQRKEAK.S...D.. 292
Rabbit Fast Skeletal ELEDMNQKLF.LR...KR.P..R.RMSADAML.AL.GSKHKVCM.L.A.. 141

Pifuc-TnI            KHKE                                        286
Japanese scallop     .SSREHEADKEGGEGENE                          293
Yesso scallop        .SSSKHAVDEEGGEGENE                          293
Yesso scallop 2      .SSSKHAVDEEGGEGEGEAENE                      314
Rabbit Fast Skeletal .QVKKEDTEKERDLRDVGDWRKNIEEKSGMEGRKKMFESES 182
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Figure 2. Multiple sequence alignments of the determined *Pinctada fucata* troponin I amino acid sequence to troponin I proteins from other species. Compared sequences were obtained from the DDBJ database: Japanese scallop *Chlamys nipponensis* (AB206837), Yesso scallop *Mizuhopecten yessoensis* (AB008005, AB008006), and rabbit *Oryctolagus cuniculus* (S61403). Numbers on the right represent the number of amino acid residues from the N-terminus. Gaps were inserted to optimize the sequence alignments. Dots indicate the identical residues present in both Pifuc-TnI and a particular compared sequence.

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

Pifuc-TnI was predominantly expressed in adductor phasic muscle, while weak expression was detected in adductor catch muscle, and no expression was detected in the gill, mantle, or foot (**Figure 6**). Therefore, the tissue distribution of *Pifuc-TnI* is equivalent to that of *Pifuc-TnC* [13]. These findings suggest that Tn is involved in the regulation of the phasic adductor muscle, rather than the catch adductor muscle.

4. Discussion

In this study, we have analyzed the molecular characteristics of Pifuc-TnI. As the primary sequence of Pifuc-TnI is 50% homologous to scallop TnI, it is possible that their steric structures are similar and they form Tn complexes in an analogous manner. Recombinant peptides of scallop and rabbit TnIs have been used to analyze the function of scallop TnI [23]. A scallop TnI C-terminal peptide (residues 232 - 292) was able to bind to actin-tropomyosin and inhibit actomyosin-tropomyosin Mg-ATPase, but was unable to interact with TnC. However, an N-terminal scallop peptide that contains a region homologous to residues

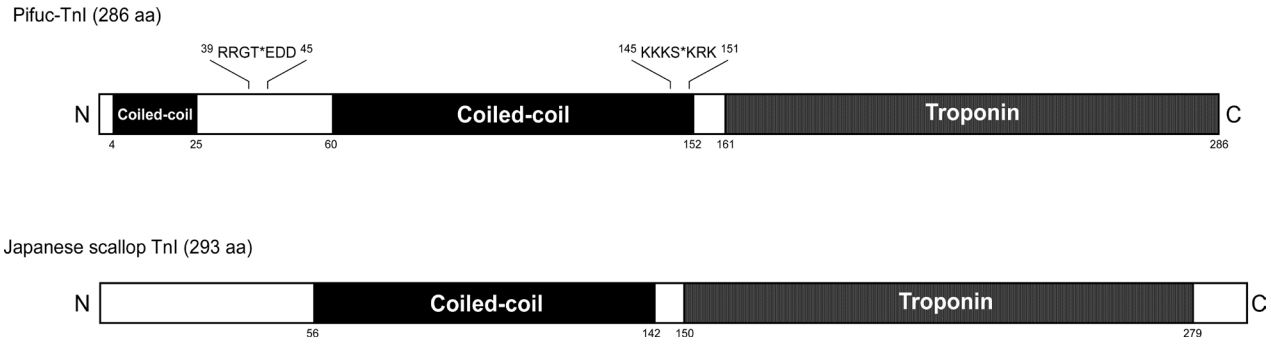

Figure 3. Motif structures of molluscan troponin I. Schematic representations of TnIs from *Pinctada fucata* and Japanese scallop *Chlamys nipponensis* showing the positions of predicted motif structures predicted by SMART, Pfam, and the Eukaryotic Linear Motif resource. Numbers represent the relative amino acid positions of each motif (from the N-terminus). RRGT*EDD and KKKS*KRK are the potential PKA phosphorylation sites in Pifuc-TnI. Asterisks indicate the phosphorylatable threonine and serine residues in each consensus sequence.

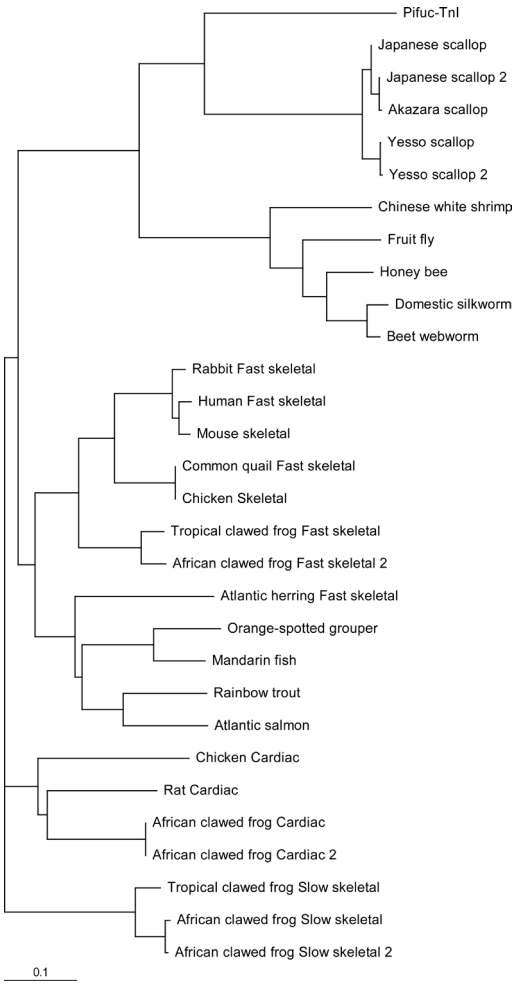

Figure 4. Phylogenetic tree showing the relationship among the troponin I amino acid sequences from *Pinctada fucata* and other species. The tree was generated using the ClustalW algorithm. The GenBank accession numbers of each sequence used in the analysis are listed in Materials and Methods.

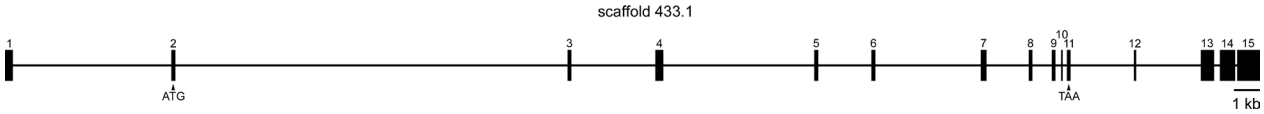

Figure 5. Gene structure of *Pinctada fucata* troponin I. Black boxes indicate exons, and intervening lines represent introns. The ATG start and TAA stop codons are indicated.

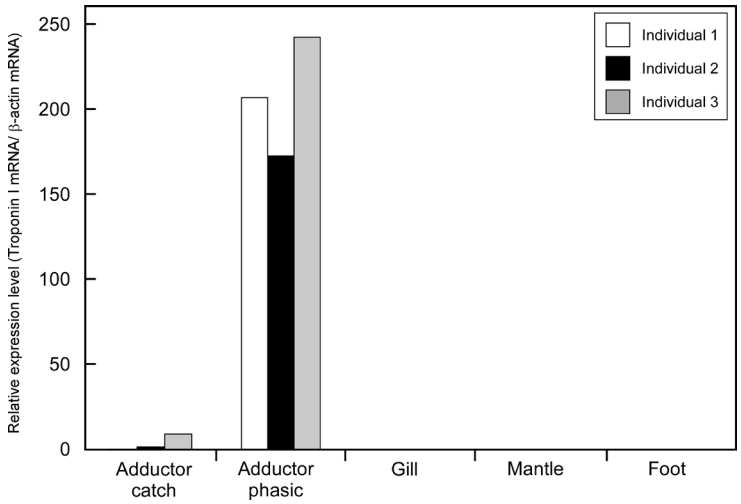

Figure 6. Gene expression patterns of troponin I in *Pinctada fucata* tissues. Quantitative real-time PCR analysis was performed to examine troponin I gene expression in *Pinctada fucata* adductor catch muscle, adductor phasic muscle, gill, mantle, and foot. The data shown are representative of three independent experiments. The y-axis indicates relative troponin I expression levels using *β-actin* as an internal standard.

1 - 30 of rabbit TnI strongly bound both TnC and actin and was able to activate the actomyosin-tropomyosin Mg-ATPase in a Ca^{2+}-dependent manner. As the C-terminal regions of Pifuc-TnI and scallop TnI are relatively homologous (**Figure 2**), we predict that the Pifuc-TnI C-terminus also does not play a role in the regulation of muscle contraction [23]. Therefore, molluscan TnI is predicted to regulate muscle contraction through an activation activity that is exerted by its N-terminal region. However, the N-terminal extending region of scallop TnI (that is not present in rabbit skeletal TnI) was shown to be unnecessary for this function and, instead is thought to play a role in maintaining the structural integrity of the Tn complex. Further studies are required to completely elucidate the role of this domain in Tn function.

TnI has been shown to be phosphorylated by several different protein kinases in vertebrate cardiac muscles [26]. We found potential PKA consensus sequences in Pifuc-TnI at residues 39 - 45 (RRGTEDD) and 145 - 151 (KKKSKRK). Intriguingly, scallop TnI does not contain any predicted PKA consensus sequences (**Figure 3**). In Pifuc-TnI, one potential phosphorylation site is located in the linker region between the two coiled-coil motifs and the other is located at the end of the second coiled-coil motif. It is possible that phosphorylation of these sites could induce a conformational change in the Pifuc-TnC molecule that al-

ters the interaction between TnI, TnT and TnC. Site-specific phosphorylation of cardiac TnI by different kinases has been revealed as a key physiological mechanism for the modulation of myofilament properties [26]. It is possible that Pifuc-Tn regulates adductor phasic muscle contraction in a similar way. PKA phosphorylation of molluscan catch muscle twitchin has been shown to abolish the catch state to relax the muscle [1]. Interestingly, PKA is able to phosphorylate twitchin proteins isolated from both scallop phasic and catch muscles *in vitro*, suggesting that both muscles utilize a signaling pathway that is dependent upon PKA phosphorylation *[27].* However, scallop TnI contains no PKA typical consensus sequences. It is certainly possible that scallop and pearl oyster adductor muscles employ different molecular mechanisms for muscle regulation. Further studies on Pifuc-TnI, particularly investigations of the effect of PKA phosphorylation on its function, are required to reveal the role of troponin in *Pinctada fucata*.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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