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The occurrence of tissue-specific twitchin isoforms in the mussel *Mytilus galloprovincialis*

Miho Kusaka¹, Daisuke Ikeda¹, Daisuke Funabara², David J Hartshorne³, and Shugo Watabe^{1,*}

¹Laboratory of Aquatic Molecular Biology and Biotechnology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo, Tokyo 113-8657

²Laboratory of Muscle Biology, Graduate School of Bioresources, Mie University, Tsu, Mie 514-8507, Japan

³Muscle Biology Group, University of Arizona, Tucson, Arizona 85721, USA

Abstract

The catch state in *Mytilus* anterior byssus retractor muscle is regulated by phosphorylation and dephosphorylation of twitchin, a member of the titin/connectin superfamily, and involves two serine residues, Ser-1075 (D1) and Ser-4316 (D2). This study was undertaken to examine whether isoforms of twitchin were expressed in various muscles of the mussel *Mytilus galloprovincialis* by reverse transcription-polymerase chain reaction. Mussel tissues, including both catch and non-catch muscles, contained various twitchin isoforms that all contained the D2 site and the kinase domain. However, sequence alterations were detected around the D1 site, notably a potential deletion of the D1 site. All isoforms from catch muscles contained both the D1 and D2 sites, whereas those from non-catch muscles also expressed the D2 site, but some of them lacked the D1 site. This suggests that the D1 site of twitchin is essential to the mechanism of catch. Genomic DNA analysis revealed that twitchin isoforms are produced by alternative splicing.

Keywords

anterior byssus retractor muscle; catch; isoform; mollusk; mussel; *Mytilus galloprovincialis*; twitchin

Introduction

Catch in molluscan adductor muscle is a state where tension is maintained for extended periods with low energy expenditure.¹ The various phases of the contraction/catch/relaxation cycle, as illustrated for anterior byssus retractor muscle (ABRM), a typical catch muscle,² involves the following events:³ stimulation of cholinergic neurons releases acetylcholine that causes an increase in intracellular $[Ca^{2+}]$ and results in rapid contraction; a reduction in $[Ca^{2+}]$ inactivates myosin and promotes the catch state; and stimulation of serotonergic neurons releases serotonin⁴ that increases $[cAMP]$ and activates the cAMP-dependent protein kinase (A-kinase) that abolishes the catch state and leads to relaxation.^{5,6} The critical role of A-kinase in this process is supported by the finding that addition of the catalytic subunit of A-kinase to permeabilized ABRM fibers releases catch.^{7,8}

*Corresponding author: Tel: 81-3-5841-7520. Fax: 81-3-5841-8166: Email: awatabe@mail.ecc.u-tokyo.ac.jp.

Several proteins were considered as possible targets for phosphorylation by A-kinase, including myosin heavy chain,⁸ myosin light chains^{9–11} and paramyosin (that is specific to and abundant in invertebrate muscles^{12,13}). However, no direct correlation was found between phosphorylation of these proteins and release of catch. Subsequently, it was shown that in both intact and permeabilized ABRM fibers the phosphorylation of a ~600 kDa protein was correlated with the release of catch.¹⁴ Characterization of the ~600 kDa protein by cDNA cloning identified it as twitchin,¹⁵ a member of titin/connectin family.¹⁶ Further investigation of the full-length cDNA revealed that *Mytilus* twitchin is composed of 23 immunoglobulin (Ig) motifs, 15 fibronectine III (FnIII) motifs and a single kinase domain.¹⁷ It was found also that three serine residues on twitchin were phosphorylated by A-kinase.¹⁸ Two of the sites were identified and named D1 (Ser-1075) and D2 (Ser-4316).^{17,18} These are located between the 7th and 8th Ig motifs, and between the 21st and 22nd Ig motifs, respectively. Phosphorylation of both D1 and D2 was proposed to be essential for the release of catch, although it appeared that the level of D2 phosphorylation was more sensitive to the transition between the catch and relaxed states.¹⁷ The third phosphorylation site, DX, tentatively was identified (from the presence of a consensus substrate sequence for A-kinase) in the region between the 7th and 8th Ig motifs and close to the D1 site.¹⁷

As mentioned above, twitchin belongs to the titin/connectin superfamily, and within this diverse group there are precedents for the existence of isoforms. For example, the twitchins from *Mytilus* and *Caenorhabditis elegans* differ in the structure of N- and C-terminal domains.^{19,20} Connectin, also known as titin, spans the separation between the Z-disc and M-line and mediates passive tension of mammalian skeletal and cardiac muscles.²¹ Like twitchin, it is composed of Ig motifs, FnIII motifs and a kinase domain.²² In addition, connectin has elastic, spring-like features, in which PEVK sequences (rich in proline, glutamine, valine, lysine) play an important role.²³ *Mytilus* twitchin also has a PEVK-like sequence between the 3rd and 4th Ig motifs.¹⁷ Connectin is encoded by a single gene and multiple isoforms are produced by alternative splicing.^{24,25} Projectin, another member of the titin/connectin superfamily contained in arthropod muscles,^{26–29} has tissue-specific isoforms of different molecular sizes.³⁰ On the other hand, whether different isoforms of twitchin can be detected in the same organism is not known.

The present study was undertaken to examine whether or not *Mytilus* twitchin isoforms could be detected in *M. galloprovincialis* and if so which isoform(s) would be specific to catch muscle. It was found that different isoforms are expressed by alternative splicing and that catch and non-catch muscles contain distinct isoforms.

Materials and Methods

Materials

Live specimens of the mussel *Mytilus galloprovincialis* (approx. 40 g including shells) were purchased from the Tokyo Central Wholesale Market. ABRM, posterior byssus retractor muscle (PBRM), mantle, gonad, adductor muscle, foot, labial pulp and gill were dissected, immediately frozen in liquid nitrogen and stored at –80°C until use.

Expression analysis

Primers specific to *Mytilus* twitchin cDNA (in the DDBJ/EMBL/GenBank databases accession number AB062881)¹⁷ were designed and used for first-strand cDNA synthesis and reverse transcription-polymerase chain reaction (RT-PCR). All tissues were dissected from three individuals and the same tissues were combined and used as one sample for preparation of total RNA.

Total RNA were extracted with ISOGEN (Nippon Gene, Tokyo, Japan) and first-strand cDNA was synthesized with appropriate primers, described below, using SuperScript III (Invitrogen, Carlsbad, CA, USA). Expression analysis of the D2 site and the kinase domain was performed using first-strand cDNA synthesized with a universal adapter primer (with poly T), whereas that of the D1 site located approximately 13 kbp upstream from the poly A tail was performed using first-strand cDNA synthesized with a gene specific primer of tw_D1_11r. Twitchin transcripts encoding the D1 and D2 sites, and kinase domain were amplified with primer pairs of tw_D1_11f plus tw_D1_11r, tw_D2_21f plus tw_D2_21r, and tw_K_F plus tw_K_R, respectively. Actin transcripts used as the internal control were amplified with a primer pair of Actin F and Actin R referring to the DDBJ/EMBL/GenBank databases accession number AF157491. Primers used in the above mentioned first-strand cDNA synthesis and RT-PCR are shown in Table 1.

Polymerase chain reaction (PCR) was carried out with denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 60 s for the D1 and D2 sites, or 90 s for the kinase domain with the final extension at 72°C for 5 min using Ex-Taq DNA polymerase (TaKaRa, Ohtsu, Japan). The PCR products obtained were applied to 1% agarose gel electrophoresis and the gel stained with ethidium bromide.

The DNA nucleotide sequences were determined with a ABI PRISM 3100 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) for the PCR products from catch and non-catch muscles after subcloning into plasmid vector pGEM-T (Promega, Madison, WI, USA).

Genomic DNA analysis

DNA was extracted using a standard phenol/chloroform method from the gonad of a single individual. Primer pairs of tw_D1_12f plus tw_D1_7r, tw_D1_9f plus tw_D1_11r, and tw_D1_11f-3 plus tw_D1_12r were used for PCR amplification (Table 1, see Fig. 3 for primer locations). Using Ex-Taq DNA polymerase and a primer pair of tw_D1_9f plus tw_D1_11r, PCR was carried out with denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 5 min with the final extension at 72°C for 5 min. Since PCR products could not be obtained by using Ex-Taq DNA polymerase and primer pairs of tw_D1_12f plus tw_D1_7r and tw_D1_11f-3 plus tw_D1_12r, PCR was carried out using LA-Taq DNA polymerase (TaKaRa) and the same primer pairs with denaturation at 94°C for 1 min, followed by 30 cycles of two steps of denaturation at 98°C for 5 s and extension at 68°C for 15 min with the final extension at 68°C for 10 min.

All PCR products were subcloned into pCR-XL-TOPO vector (Invitrogen). At first, the nucleotide sequence of PCR products amplified with primer tw_D1_9f and tw_D1_11r was determined by the primer walking method. Since shotgun sequencing was found to be more efficient than primer walking, PCR products amplified with primer pairs of tw_D1_12f plus tw_D1_7r and tw_D1_11f-3 plus tw_D1_12r were then subjected to shotgun sequencing described below.

Shotgun sequencing

PCR products subcloned into pCR-XL-TOPO vector were cut into short fragments with Nebulizers (Invitrogen). These fragments were subcloned into pUC118 vector using a blunt end ligation kit (TaKaRa) and sequenced as described above. The Phred/Phrap/Consed^{31–33} sequence analysis software package was used. Sequences determined were processed using Phred, trimmed for quality, screened for vector sequences and assembled using Phrap. Quality scores were assigned automatically and the electropherograms and assembly were viewed and verified for accuracy using Consed.

Results

Expression analysis

Figure 1 shows the tissue-specific expression patterns of transcripts encoding the D1 and D2 sites and the kinase domain from various tissues. Catch muscles, including ABRM, PBRM and adductor muscle, showed high levels of expression for the above three types of transcripts, whereas non-muscle tissues such as gonads, labial pulp and gills showed only marginal levels of expression. Non-catch muscles, including mantle and foot tissues, exhibited intermediate levels of expression between the catch muscles and non-muscle tissues, suggesting that mantle and foot contain some muscle tissues. Interestingly, PCR products encoding the region covering the D1 site contained fragments of ~210 bp for gonad, mantle and foot, in addition to that of the expected size, ~400 bp. On the other hand, ABRM, PBRM and adductor muscle showed high expression levels with only the 400 bp fragment. These analyses were repeated three times and reproducible results were obtained.

Sequences of PCR products

PCR products from catch and non-catch muscles (Fig. 1) were subcloned and subjected to the sequence analysis. The PCR products encoding the D1 site contained sequences with a 42 bp insertion, a 15 bp deletion and a 186 bp deletion in comparison with the published *Mytilus* twitchin cDNA sequence (AB062881).¹⁷ The insert and two deleted sequences were named R1, R2 and R3, respectively (Fig. 2). R3 encodes the D1 site, thus those clones without R3 lack the D1 site. Several experiments were repeated for subcloning and the distribution of R1, R2 and R3 in various muscles are summarized in Table 2. A striking feature is that although the distribution of the individual regions varied, all of the catch muscles contained the R3 region and those clones without R3 were derived from the non-catch muscles of mantle and foot (Table 2). (One clone without R3 was obtained from PBRM and this was probably due to contamination by non-muscle tissues during RNA extraction). The decreased size due to the deletion of R3 was consistent with the results obtained by RT-PCR for expression analysis (Fig. 1). It was impossible to distinguish the different transcripts regarding R1 and R2 in the electrophoresis. On the other hand, regions encoding the D2 site and the kinase domain contained no insertions or deletions (data not shown) and were the same as the sequence published before.¹⁷

Genomic DNA sequence encoding the D1 site

PCR was performed with genomic DNA extracted from gonads and specific primers for the D1 site (Table 1). Three fragments were obtained; a 5 kbp fragment amplified with a primer pair of tw_D1_12f plus tw_D1_7r that contained R1, a 6 kbp fragment amplified with a primer pair of tw_D1_9f plus tw_D1_11r that contained R2 and R3, and a 10 kbp fragment amplified with a primer pair of tw_D1_11f-3 plus tw_D1_12r that contained R1, R2 and a part of R3 (Fig. 3a).

The 5 kbp fragment containing R1 was analyzed for partial sequences of approximately 600 bp each from the 3' and 5' ends for 16 clones, resulting in no difference in the intron sequence. One clone was subjected to shotgun sequencing to determine its full sequence. R1 was encoded by exon R1-1 or R1-2, and the two sequences of 2223 bp containing exon R1-1 or R1-2 together with the succeeding intron were repeated in tandem. Identities of the two sequences were high (96.8%) (Fig. 3b). In eukaryotes, it is known that the intron sequences donate conserved sequences for RNA splicing, including the 5' splice, branch and 3' splice sites.³⁴ Such sequences flanking the two entire exons R1-1 and R1-2 were identical and their 5' splice, branch and 3' splice sites were conserved (Fig. 4a), suggesting that both exons encoding R1 are expressed.

Thirty clones of the 6 kbp fragment containing R2 and R3 were analyzed by the primer walking method. R2 and R3 were encoded by different exons and two types of clones (types A and B), attributed to different intron sequences (Fig. 3b), were obtained at a ratio of 1:1. Their identity was 96% and the partial sequences (754–1294) in which most differences were found, are shown in Figure 4b.

The 10 kbp fragment containing R1, R2 and part of R3 also was sequenced for 600 bp from both the 3' and 5' ends for 16 clones, resulting in no difference in the intron sequence. One clone was analyzed for its full-length sequence by shotgun sequencing, yielding the exon-intron structure outlined in Figure 3b and putative splicing patterns shown in Figure 5. This fragment contained the type A sequence for the 6 kbp fragment (Figs 3b and 4a).

Discussion

Expression analysis of twitchin transcripts encoding the D1 and D2 sites and the kinase domain was carried out. Similar differences in the expression levels among various tissues were observed each for the transcripts encoding the D1 and D2 sites and the kinase domain. The expression levels were high in catch muscles (ABRM, PBRM and adductor muscle), low in non-catch muscles (foot and mantle) and marginal in non-muscle tissues (gonad, labial pulp and gill) (see Fig. 1). Two types of clones (types A and B) attributed to different intron sequences (Fig. 3b) were found and considered to be derived from a variation in the allelic gene, since the heterogeneity was small and no sequence difference was observed in the connecting exons. Although twitchin is an important protein regulating catch contraction, its function(s) in non-catch muscle and non-muscle tissues is not established.

The physiological roles of other members of the superfamily, to which twitchin belongs, are being resolved. An example is connectin. It is proposed that connectin maintains sarcomere structure and controls passive tension.²¹ Sarcomere assembly was inhibited with morpholino antisense oligonucleotides specific for the N2A and N2B regions of zebrafish connectin.³⁵ A signaling pathway was identified for mammalian connectins that involved the kinase domain and its interaction with the zinc-finger protein, nbr1.³⁶ Nbr1 interacts with p62, which in turn interacts with MuRF2, a muscle-specific RING-B-box protein. MuRF2 is the ligand of the transactivation domain of the serum response transcription factor. It is suggested that gene expression of muscle specific proteins are controlled via this pathway.³⁶ To date, no function or role has been ascribed to the kinase domain of twitchin in the catch mechanism, although a physiological role in other cell processes remains a possibility. The presence of twitchin in non-muscle tissue, although low, raises the question whether twitchin has other functions besides being a regulator of the contractile process.

Sequence analyses of the RT-PCR products encoding the D2 site and the kinase domain were identical, whereas those encoding the D1 site showed several distinct sequences. In comparison to the published twitchin sequence,¹⁷ the D1 transcripts showed an insertion (R1) and two deletions (R2 and R3). Genomic DNA analyses demonstrated that these variations are produced by alternative splicing (see Figs 3b and 5), and to the authors' knowledge this is the first report of twitchin isoforms (i.e. in a single organism). The isoforms containing R1 and/or R2 were obtained from both catch and non-catch muscles. However, the isoform with the R3 deletion (i.e. the D1 site) was found only in non-catch muscles (see Table 2 and Fig. 5b).

The function(s) of the R1 and R2 regions in twitchin isoforms are not clear. However, these regions appear not to be critical for the catch mechanism, since twitchin from catch muscles contains R3 but not always R1 or R2. Connectin contains numerous isoforms produced by alternative splicing in the same tissue, and distinct roles for these isoforms are not identified.³⁷ Several muscle proteins have tissue-specific isoforms containing A-kinase phosphorylation

sites. The cardiac-specific isoforms of troponin I,³⁸ myosin binding protein C^{39,40} and connectin⁴¹ are targets of A-kinase. Phosphorylation of these proteins by A-kinase following adrenergic stimulation modifies various aspects of cardiac function.^{42–44}

An important point raised in the present study is that the D1 site of twitchin is always found in catch muscles. It was suggested previously that both the D1 and D2 A-kinase sites are essential for the catch mechanism.¹⁷ In a recent study, the role of the D2 site was investigated. It was proposed that at least part of the mechanical connection in catch is due to the phosphorylation-dependent binding of the D2 peptide (represented by the D2 site flanked by two Ig motifs) to both actin and myosin.⁴⁵ The actin-D2 link is suggested to be competitive with the actin–myosin interaction (cross bridge) and thus catch is promoted under the low force state (i.e. at lower $[Ca^{2+}]$). It was also proposed that twitchin could modulate the contractile cycle in molluscan striated muscle, notably the relaxation phase,⁴⁶ and this is consistent with the presence of the D2 site of twitchin in both catch and non-catch muscle. Obviously, the emphasis now is to characterize those interactions involving the D1 site. It appears that the D1 and D2 sites have separable functions in the catch process, but this must be resolved.

In the present study, RT-PCR was performed and focused on the regions encoding the D1 and D2 sites and kinase domain. As a result, it was found that *Mytilus* twitchin contained isoforms the transcripts of which were produced by alternative splicing. The transcripts without the D1 site, where Ser-1075 is phosphorylatable, were not expressed in catch muscles, suggesting that the D1 site plays an important role in these muscles.

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Fig. 1.

Reverse transcription-polymerase chain reaction analysis of different regions of the twitchin gene for various tissues from *Mytilus galloprovincialis*. The twitchin gene was analyzed in regions encoding the D1 and D2 sites and the kinase domain, using the actin gene as control. M, molecular weight markers; ABRM, anterior byssus retractor muscle; PBRM, posterior byssus retractor muscle; adductor, adductor muscle; labial, labial pulp.

Fig. 2.

Nucleotide and deduced amino acid sequences of twitchin cDNA. (a) Nucleotide sequences of polymerase chain reaction amplification products encoding the D1 phosphorylation site of twitchin from *Mytilus galloprovincialis* anterior byssus retractor muscle (ABRM) and mantle. Nucleotides identical to those of the published *Mytilus* twitchin cDNA sequence¹⁷ (accession no. AB062881) are shown by dots. Bold letters indicate the codon for the A-kinase site (Ser-1075). Underlines indicate primer sequences. Numbers next to 'ABRM' and 'Mantle' indicate the clone number. Numbers to the right of the sequences are the nucleotide numbers for the cDNA clones. (b) Deduced amino acid sequences from ABRM and mantle. Amino acids identical to those of the published *Mytilus* twitchin¹⁷ are shown by dots. Bold letters

indicate the D1 A-kinase site (Ser-1075). Numbers to the right of the sequences indicate the number of amino acids in the cDNA clones. Boxes of R1, R2 and R3 in (a) and (b) indicate those sequences which are either inserted or deleted in comparison with the published sequence. cDNA sequences of ABRM4, ABRM7, Mantle 1 and Mantle 3 have been registered to the DDBJ/EMBL/GenBank databases with accession numbers AB433987, AB433988, AB433989 and AB433990, respectively.

Fig. 3.

The exon-intron structure of genomic DNA encoding the *Mytilus galloprovincialis* twitchin in the region of the 7th to 8th Ig motif. (a) Primer positions on cDNA used for amplification of genomic DNA. Arrows indicate primer positions. Shaded boxes represent parts of the Ig motifs. R1, R2 and R3 are the sequences which are deleted or inserted in comparison with the published sequence of *Mytilus* twitchin cDNA.¹⁷ (b) Predicted exon-intron structure of genomic DNA in the region of the 7th to 8th Ig motif. Exons and introns are represented by filled boxes and solid lines, respectively. The dashed box shows the location of the variable sequences, shown in Figure 4b. The locations of the 5, 6 and 10 kbp fragments amplified with the polymerase chain reaction primers, shown in panel (a), are presented under the exon-intron structure. Arrowheads in panels (a) and (b) indicate the location of the D1 A-kinase site (Ser-1075). The sequences of 5 kbp fragment, 6 kbp fragment type A, 6 kbp fragment type B and 10 kbp fragment have been registered to the DDBJ/EMBL/GenBank databases with accession numbers of AB433991, AB433992, AB433993 and AB433994, respectively.

(a)

R1-1	GTCTTTCA CTGACT CGTTTATTTTCACTCATTTTGTTCATATGAAATTGATATGTACTT	528
R1-2	GTCTTTCA CTGACT CATTTTATTTTCACTCATTTTGTTCGTTATGAAATTGATATGTACTT	2753
R1-1	TTTTT CAG TGGAAGGCCAAGACCAAGCCAACTGCAAAAACACCGCAG GTATGTAAAG	588
R1-2	TTTTT CAG TGGAAGGCCAAGACCAAGCCAACTGCAAAAACACCGCAG GTATGTAAAG	2813

(b)

Type A	TACTTCATCCACTGGCTCTGATGTCATGGGGGATTTCTTTAATGTTCAATTACGTCACAG	814
Type B	TACTTCATCCACTGGCTCCGATGTCCAGGCCGATTTCTTTAATGTTCAATTACGTCACAG	838

Type A	AGATATGACTAAAGACCAGGCAATTGATTCTGTGTGATTTTTTTTATAGTGCTGCTGATA	874
Type B	AGATATGACTAAAGACCAGGCAATTGATTCTGTGTGATTTTTTTTATAGTGCTGCTGATA	898

Type A	AAATATTGAAACATGCTACAAGAATAAGAGTAGATTTTTTGTTCCTTGCTTTTCAAAATT	934
Type B	AAATATTGAAACATGCTACAAGAATAAGAGTAGATTTTTTGTTCCTTGCTTTTCAAAATT	958

Type A	ATAATTTAGAATAATTTTAAATAGGAATTCCTATTGGAATTTTGAAACAAGGATAGAA	994
Type B	ATAATTT-----TAGAA	970

Type A	TAATTTTGCTAAAGTAGGTACTTGAATATGTTTAATACACAATTATGTACTCTTTTCATT	1054
Type B	TAATTTTGCTAAAGTA---CTTGAATATGTTTAATACACAATTATGTACTCTTTTCATT	1026

Type A	GTTTATAATTTGAAAGCCGTACTTTTATTATTAAATGTAGAAAGTCTTATAACAACAT	1114
Type B	GTTTATAATTTGAAAGCCATACCTTTTATTATTATA-----	1061

Type A	TATTTATTTTATTAAGATCAAATAGAAATATTTTATTGACCTATTGTGATCGGTAAAAAA	1174
Type B	-----GATCAAATAGAAATATTTTATTGACCTATTGTGATCGGTAAAAAA	1107

Type A	TGTTAGCAGAACATCTACTCTTAATTTTGAGTATTTCAAAAATGAGTAGGCAATAATGAC	1234
Type B	TGTTGGCAGAACATCTACTCTTAATTTTGAGTATTTCAAAAATGAGTAGGCAATAATGAC	1167

Type A	ATATTAGATAATTGAGGTGATATTTGCTAGAGTAGAAATATATATGGTAGAGCTGAATTT	1294
Type B	TTATTAGATAATTGAGGTGATATTTGCTAGAGTAGAAATATATATGATAGAGCTGAATTT	1227

Fig. 4.

Partial sequences of genomic DNA of the *Mytilus galloprovincialis* twitchin. (a) Conserved nucleotide sequences in the 3' and 5' splicing and branch sites of introns flanking the R1 exons. The shaded box indicates the exon encoding R1-1 and R1-2. Bold letters indicate conserved nucleotides in the splicing and branch sites. Numbers to the right of the sequences indicate those in the 5 kbp fragment. (b) Partial sequence of the 6 kbp fragment, containing R2 and R3, amplified with a primer pair of tw_D1_9f plus tw_D1_11r. This fragment contained two types of intron sequences. Asterisks indicate identical nucleotides. The location of the sequences shown in this figure are represented by the dashed box in Figure 3b. Numbers to the right of the sequences indicate those in the 6 kbp fragment.

Fig. 5.

Alternative splicing patterns of the *Mytilus galloprovincialis* twitchin gene in the region encoding the 7th to 8th Ig motif. (a) The exon-intron structure encoding the region of the 7th to 8th Ig motif. The arrowhead indicates Ser-1075 in the D1 site. (b) Transcripts expressed in catch and non-catch muscles. (c) Transcripts expressed only in non-catch muscles.

Table 1

Primers used in the experiments

Primer name	Sequence
First-strand cDNA synthesis	
adapter primer	5'-GGCCACGCGTCGACTAGTAC(T)17-3'
tw_D1_11r [†]	5'-TCCAATTCAACAATGTACTTGTCTGG-3'
RT-PCR	
Forward primer	
tw_D1_11f	5'-TAAAGATCTTAAAGATTGAGCTATGG-3'
tw_D2_21f	5'-CAAATACATGCAAAAATACAGAGGCA-3'
tw_K_F	5'-CCTCAGCCTGTTTCAGTTAGG-3'
actin F	5'-AAGTCAGGGTGAGATACTCC-3'
Reverse primer	
tw_D2_21r	5'-GACTGTACCTTTATAGTGGCGACACC-3'
tw_K_R	5'-AGCGACTAGATGGAATTCTGG-3'
actin R	5'-GTTGTAGAAGGTGTGATGCC-3'
Long PCR for genomic DNA analysis	
Forward primer	
tw_D1_12f	5'-GATGATGATATTGACTGGGGG-3'
tw_D1_9f	5'-GAAAGAACGACGTCCATCTTTGG-3'
tw_D1_11f-3	5'-CACAGTTATTGTTGAACAAGAAGG-3'
Reverse primer	
tw_D1_7r	5'-ATCAACCAAAGATGGACGTCG-3'
tw_D1_12r	5'-CTCGGGATAGTTTTCTCATGG-3'

[†] Used as a reverse primer for reverse transcription-polymerase chain reaction (RT-PCR) and PCR for genomic DNA analysis.

Frequency of polymerase chain reaction products containing deletions and an insertion for the *Mytilus galloprovincialis* twitchin gene

Region	Frequency of PCR done							
	R1	R2	R3	ABRM	PBRM	Adductor	Mantle	Foot
+	+	+	+	3	0	1	3	1
+	-	-	+	0	0	2	0	0
-	+	+	+	12	0	0	1	0
-	-	-	+	3	5	4	0	0
+	+	+	-	0	0	0	0	1
+	-	-	-	0	0	0	0	0
-	+	+	-	0	0	0	2	0
-	-	-	-	0	1	0	2	5

The presence (+) or absence (-) of the relevant R region is indicated.
ABRM, anterior byssus retractor muscle; PCR, polymerase chain reaction; PBRM, posterior byssus retractor muscle.