RESEARCH ARTICLE

Cloning of Skeletal Myosin Heavy Chain Gene Family From Adult Pleopod Muscle and Whole Larvae of Shrimps

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ABSTRACT

The physiological and biological properties of skeletal muscle in crustacea have not been well understood compared with those of vertebrates. The present study focused on myosin, the major protein in skeletal muscle, from shrimps. In our previous study, two full-length genes encoding myosin heavy chain (MHC), a large subunit of the myosin molecule, were cloned from abdominal fast skeletal muscle of kuruma *Marsupenaeus japonicus*, black tiger *Penaeus monodon* and Pacific white *Penaeus vannamei* shrimps, and named as MHCa and MHCb. In this study, we renamed these as MHC1 and MHC2, respectively, due to the presence of various isoforms newly identified. Partial MHC sequences were identified from pleopod muscle of these shrimps. Two MHCs, named MHC3 and MHC4, were identified from pleopod muscle of kuruma shrimp, whereas two MHCs, named MHC4 and MHC5, were cloned from Pacific white shrimp pleopod. MHC3 was cloned only from black tiger shrimp pleopod. Partial MHC sequences from zoea, mysis, and postlarvae of black tiger and Pacific white shrimps were also determined. The phylogenetic tree demonstrated that most MHCs from pleopod muscle and larval MHCs formed clades with MHC1 and MHC2, respectively. These MHCs were considered to be of fast type, since MHC1 and MHC2 are fast-type MHCs according to our previous study. MHC5 obtained from pleopod muscle of Pacific white shrimp in this study was monophyletic with American lobster *Homarus americanus* S_2 slow tonic MHC previously reported, indicating that MHC5 from Pacific white shrimp is of slow type. *J. Exp. Zool. 319A:268–276, 2013.* © 2013 Wiley Periodicals, Inc.

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Myosin, the major protein in skeletal muscle, is a hexamer composed of two heavy chain subunits (myosin heavy chain, MHC) each with approximately 200 kDa, and four light chain subunits (myosin light chain, MLC) each with approximately 20 kDa (Harrington and Rodgers, '84). The myosin consists of a globular head called subfragment-1 (S1) at the N-terminal half and a coiled-coil structure of α-helices called rod at the C-terminal half. S1 is composed of two heavy chains each associated with two MLCs and each S1 heavy chain consists of three domains of 25, 50,

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and 20 kDa in the order from the N terminus (Balint et al., '78; Mornet et al., '79; Rayment et al., '93). These domains are produced by limited proteolysis at two structurally flexible regions called loop 1 and loop 2. While S1 has physiologically important functions such as ATP and actin binding (Lowey et al., '69; Harrington and Rodgers, '84; Cope et al., '96; Bobkov et al., '97), loop 1 participates in the sliding of myosin-containing thick filaments on actin-containing filaments. On the other hand, loop 2 functions in actin-activated myosin Mg^{2+}-ATPase (Uyeda et al., '94; Murphy and Spudich, '98, '99; Sweeney et al., '98). The myosin rod consists of two domains called subfragment-2 (S2) and light meromyosin (LMM) at the N- and C-terminal sites, respectively. The rod transduces chemical energy produced by S1 ATPase into mechanical energy during sliding of thick filaments on thin filaments, resulting in muscle contraction (Harrington and Rodgers, '84).

MHC isoforms possess different primary structures with different expression patterns. For instance, mammals have six fast skeletal MHCs. Embryonic and perinatal MHCs are expressed during pre- and postnatal development of skeletal muscle, respectively, whereas IIa, IIb, and IId/x MHCs, primarily in adult fast skeletal muscles with oxidative (IIa) and glycolytic (IIb and IId/x) metabolism (Lyons et al., '90; DeNardi et al., '93; Schiaffino and Reggiani, '96; Schiaffino and Salviati, '97). Type II MHC is expressed in fast fibers exerting rapid contraction, whereas type I MHC is expressed in slow fibers which have slow contraction speed (Bassel-Duby and Olson, 2006).

Although crustacea form a large, diverse group, the physiology and biochemistry of their skeletal muscle have been poorly understood compared with vertebrate counterparts. Although only fragmental information is available about invertebrate MHCs (Hooper and Thuma, 2005; Hooper et al., 2008), MHCs from crustacea have been studied to some extent and certain important data are available which characterize crustacean muscle. For instance, NADH-diaphorase and ATPase activity stainings were carried out in abdominal muscle of American lobster *Homarus americanus* (Ogonowski and Lang, '79). According to the data, superficial extensor muscle, superficial flexor muscle, pleopod muscle and their neighbors belong to slow muscle, and remaining parts do to fast muscle. It has also been reported that the ratio of fast muscle fiber to slow muscle fiber in the cutter claw of American lobster increases in a growth-dependent manner (Mykles, '97). Partial MHC sequences were determined for American lobster, where three MHCs were identified from different muscles and named fast, S_1 slow twitch and S_2 slow tonic MHCs (Cotton and Mykles, '93; Medler and Mykles, 2003; Medler et al., 2004). Ghost crab *Ocypode quadrata* is known to have at least three MHCs and their sequences were partially determined, although their fiber types have not been identified yet (Perry et al., 2009). Information about crustacean MHCs expressed in larvae is also available. The amino acid sequences of loop 1 regions of European lobster *Homarus gammarus* MHCs expressed in zoea and mysis were determined (Magnay et al., 2003).

To shed light on crustacean skeletal muscle as a comparative model with vertebrate and other invertebrate counterparts, it is useful to investigate primary structures, expression patterns, and tissue distributions of MHCs in shrimps. We reported previously the complete sequences of two MHC genes (*MHC*s), *MHCa* and *MHCb*, from abdominal muscles of adult specimens of kuruma *Marsupenaeus japonicus*, black tiger *Penaeus monodon* and Pacific white *Penaeus vannnamei* shrimps (Koyama et al., 2012a, b). *MHCa* transcripts were localized in flexor muscle, whereas *MHCb* transcripts were in extensor and flexor muscles. Both *MHCa* and *MHCb* transcripts were not localized in pleopod muscle.

The present study was undertaken to clone *MHC*s from adult pleopod muscle of the above-mentioned three shrimp species and from the whole larvae and postlarvae of black tiger and Pacific white shrimps, revealing that shrimps contain unexpectedly diverse types of skeletal muscle MHCs.

MATERIALS AND METHODS

Shrimps

Kuruma shrimp were obtained from Miyazaki Prefecture, Japan, and black tiger and Pacific white shrimps were obtained from Thailand. Adult shrimps were instantly killed by cutting the ventral nerve cord and their pleopods were collected and preserved in RNA*later* (Ambion, Austin, TX, USA) until RNA extraction. Larvae and postlarvae of black tiger and Pacific white shrimps were also preserved in RNA*later* until RNA extraction.

Designing of Primers for Amplification of Shrimp *MHC*s

CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primers) (Rose et al., '98) was used to design degenerate primer F (Table 1) based on the deduced amino acid sequences of MHC from fruit fly *Drosophila melanogaster* (DDBJ/EMBL/GenBank accession number AAA28686) and American lobster fast- (U03091) and S_1 slow twitch-type (AY232598) MHCs. This primer has been demonstrated to amplify DNA fragments encoding MHCs from abdominal muscle of kuruma, black tiger, and Pacific white shrimps as reported previously (Koyama et al., 2012a,b).

Cloning of *MHC*s From Pleopod Muscle

Total RNAs were extracted from pleopod muscle of kuruma shrimp using ISOGEN solution (Nippon Gene, Tokyo, Japan) according to

Table 1. Primer sequences to amplify myosin heavy chain genes from shrimps.

Name	Sequence (5′–3′)
F	GGCCCTGCGCATGAARAARAARYT
GeneRacer 3′ primer	GCTGTCAACGATACGCTACGTAACG

the manufacturer's instructions. First strand cDNA synthesis was performed using a GeneRacer Oligo dT Primer [5′-GCTGTCAACGATACGCTACGTAACGGCATGACAGTG(T)$_{24}$-3′] (Invitrogen, Carlsbad, CA, USA) and Superscript III reverse transcriptase (Invitrogen).

The 3′-region of *MHC* was amplified by 3′ rapid amplification of cDNA ends using degenerate primer F (Table 1) and GeneRacer 3′ Primer (Invitrogen, Table 1). PCR was performed using KOD-Plus-Neo DNA polymerase (Toyobo, Osaka, Japan) with denaturation at 94°C for 2 min followed by 40 cycles of 98°C for 10 sec, 55°C for 30 sec, and 68°C for 1 min. The amplified PCR products were subcloned into a plasmid, pBluescript II KS (+) vector (Stratagene, La Jolla, CA, USA), and sequenced by ABI PRISM DNA sequencer model 3100 using BigDye terminator cycle sequencing kit ver. 3.1 (Applied Biosystems, Foster City, CA, USA).

The same procedure was employed to determine the partial sequences of pleopod MHCs from black tiger and Pacific white shrimps.

Cloning of *MHC*s From Larvae and Postlarvae of Black Tiger and Pacific White Shrimps

Larvae at nauplius, zoea, and mysis stages and postlarvae (Motoh, '79) were used as samples for black tiger shrimp. Larvae at all stages except nauplius of Pacific white shrimp were also used. In the case of Pacific white shrimp, samples at two different postlarval stages were examined, one sample at 3 days post-metamorphosis (dpm) and the other at 20 dpm.

The methods to determine the sequences of MHCs from larvae and postlarvae are described above.

Phylogenetic Analysis

The deduced amino acid sequences of kuruma shrimp, black tiger shrimp, and Pacific white shrimp MHCs were aligned by Clustal W (Thompson et al., '94). Since only C-terminal sites have been reported for American lobster and ghost crab MHCs used as references, the corresponding sequences were used to construct a phylogenetic tree by the neighbor-joining method using MEGA 4.1 (Kumar et al., 2008) in comparison with those of American lobster, ghost crab, two fly species *D. melanogaster* and *D. virilis*, scallop *Argopecten irradians*, two squids *Loligo pealei* and *L. bleekeri* and common carp *Cyprinus carpio*.

RESULTS

Deduced Amino Acid Sequences of *MHC*s From Pleopod Muscle

We cloned various *MHC*s from adult pleopod muscle and the whole larvae and postlarvae at various developmental stages of the three shrimps. Thus simple nomenclature system for MHCs previously cloned from adult abdominal muscle as MHCa and MHCb was not easily applicable to MHCs newly identified in this study. Therefore, we renamed MHCa and MHCb to MHC1 and MHC2, respectively, and further to mjMHC1 and mjMHC2 for kuruma shrimp. Accordingly, MHCa and MHCb cloned from adult abdominal muscle of black tiger shrimp were renamed as pmMHC1 and pmMHC2, respectively, where those of Pacific white shrimp, pvMHC1 and pvMHC2, respectively.

We cloned two, one, and two MHCs from pleopod muscle of kuruma, black tiger, and Pacific white shrimps, respectively.

Two types of *mjMHC*s were amplified from pleopod muscle of kuruma shrimp with a primer set of primer F and GeneRacer 3′ Primer and named as *mjMHC3* (DDBJ/EMBL/GenBank accession number AB759096) and *mjMHC4* (AB759097), because their sequences were very different from those of *mjMHC1* and *mjMHC2*. The amplified products encoding mjMHC3 and mjMHC4 consisted of 976 and 1,066 bp, respectively (Fig. 1).

Only one type of *MHC* was cloned from pleopod muscle of black tiger shrimp with the same primer set used for kuruma shrimp and named as *pmMHC3* (AB759098), because its sequence was similar to that of *mjMHC3* (Fig. 1). *pmMHC3* consisted of 976 bp.

As in the case of kuruma shrimp, two types of *MHC*s were amplified from pleopod muscle of Pacific white shrimp with the same primer set. Since one type of *pvMHC* had the deduced amino acid sequence similar to that of *mjMHC4*, it was defined as *pvMHC4* (AB759099). The deduced amino acid sequence of the other *MHC* from pleopod muscle of Pacific white shrimp was markedly different from those of *MHC1*, *MHC2*, *MHC3*, and *MHC4*. Therefore this *MHC* from Pacific white shrimp was named *pvMHC5* (AB759100). The amplified products encoding pvMHC4 and pvMHC5 were 1,064 and 1,145 bp in size, respectively (Fig. 1).

The amino acid identities between MHC1 from abdominal muscle and MHCs from pleopod muscle except pvMHC5 from Pacific white shrimp were 78–89%, and those between MHC2 and MHCs from pleopod muscle except pvMHC5 were 72–75% (Table 2). The amino acid identities between MHC1 and pvMHC5 were 69–70% and those between MHC2 and pvMHC5 were 80–81% (Table 2).

Deduced Amino Acid Sequences of *MHC*s From the Whole Larvae and Postlarvae

One *MHC* was cloned each from zoea, mysis, and postlarvae of black tiger shrimp with 921, 934, and 921 bp, respectively. The three MHCs had only slightly different deduced amino acid sequences from each other (Fig. 2). Because their sequences were different from those of adult MHCs, MHCs from zoea, mysis, and postlarvae were named pmMHC2Z (AB759101), pmMHC2P (AB759102), and pmMHC2M (AB759103), respectively. Unfortunately, cDNA cloning of MHC was not successful from black tiger shrimp at nauplius stage.

The 3′-region of MHC was also cloned from zoea, mysis, and postlarvae at 3 and 20 dpm of Pacific white shrimp with the same size of 926 bp. MHC clones from zoea, mysis, and postlarva at 20 dpm had an identical deduced amino acid sequence. On the other hand, MHC from postlarva at 3 dpm of Pacific white shrimp was slightly different in the deduced amino acid sequence from

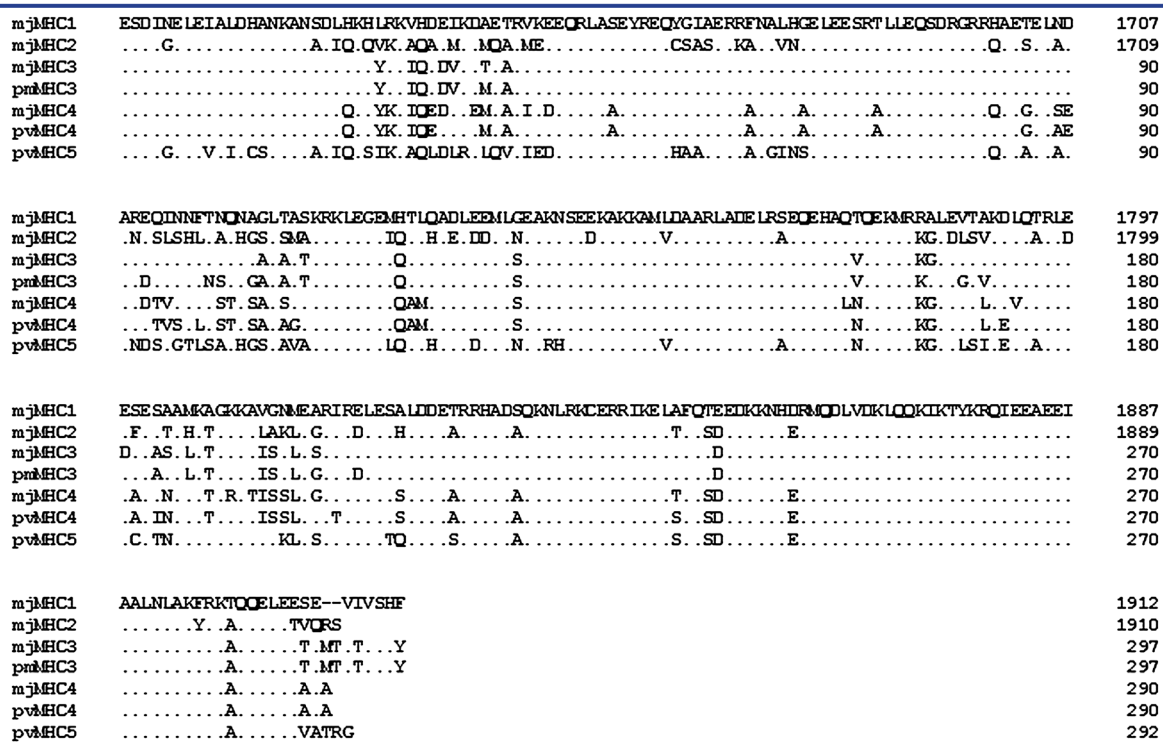

Figure 1. The C-terminal amino acid sequences of pleopod muscle myosin heavy chains from adult specimens of kuruma, black tiger, and Pacific white shrimps in comparison with those of fast abdominal muscle counterparts. Fast abdominal muscle myosin heavy chains (MHCs), MHC1 and MHC2, were cited from Koyama et al. (2012a). Amino acid residues in MHC2, MHC3, MHC4, and MHC5 identical to those in MHC1 are indicated by dots and hyphens denote deletions. Abbreviations used are: mj, kuruma shrimp *Marsupenaeus japonicus*; pm, black tiger shrimp *Penaeus monodon*; pv, Pacific white shrimp *Penaeus vannamei*.

Table 2. The amino acid identity of myosin heavy chains from abdominal and pleopod muscle of adult specimens of kuruma, black tiger, and Pacific white shrimps (%).

	Abdominal		Pleopod		Abdominal		Pleopod	Abdominal		Pleopod	
	mjMHC1	mjMHC2	mjMHC3	mjMHC4	pmMHC1	pmMHC2	pmMHC3	pvMHC1	pvMHC2	pvMHC4	pvMHC5
mjMHC1[a]		71	89	80	93	72	87	92	72	81	70
mjMHC2			73	73	71	96	74	71	96	75	80
mjMHC3				83	87	73	95	86	73	85	72
mjMHC4					79	72	84	78	72	93	73
pmMHC1[a]						71	87	96	71	81	70
pmMHC2							73	71	99	75	80
pmMHC3								86	73	84	72
pvMHC1[a]									72	80	69
pvMHC2										75	81
pvMHC4											74

[a]Abbreviations used are: MHC, myosin heavy chain; mj, kuruma shrimp *Marsupenaeus japonicus*; pm, black tiger shrimp *Penaeus monodon*; pv, Pacific white shrimp *Penaeus vannamei*.

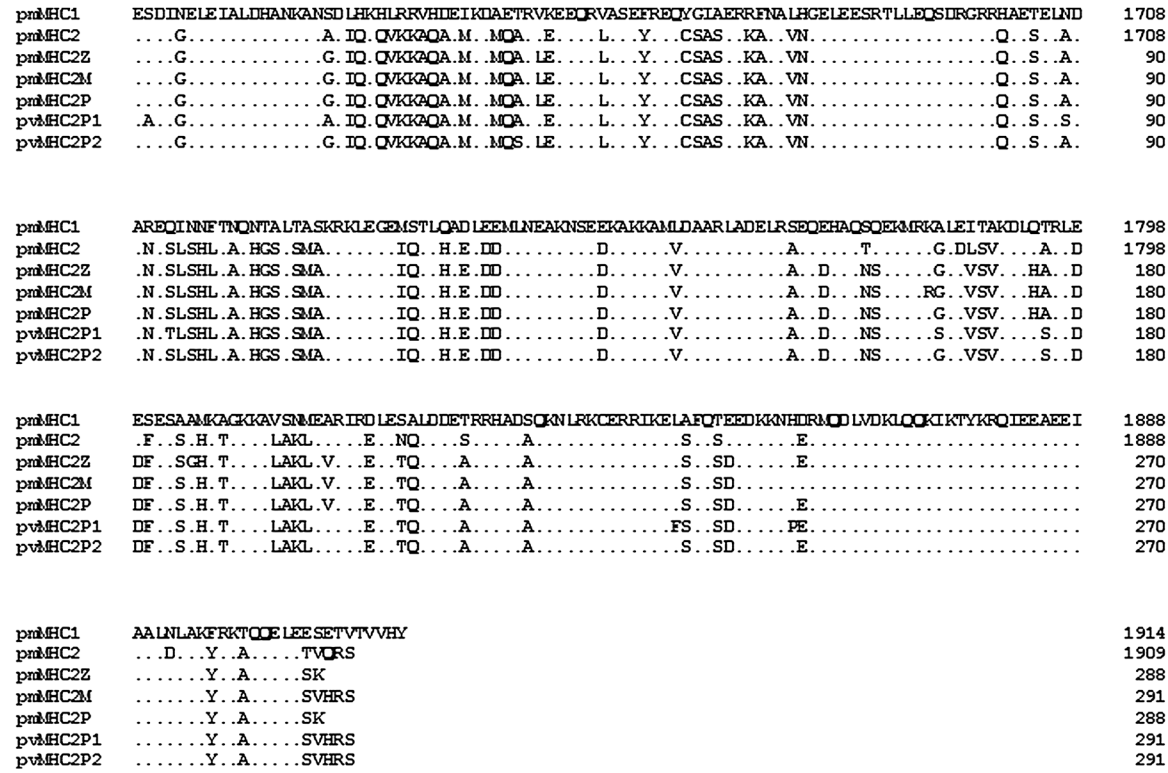

Figure 2. The C-terminal amino acid sequences of myosin heavy chains from larvae and postlarvae of black tiger shrimp and Pacific white shrimp in comparison with those of fast abdominal muscle counterparts. Fast abdominal muscle myosin heavy chains (MHCs), MHC1 and MHC2, were cited from Koyama et al. (2012b). Amino acid residues in MHC2s identical to those in MHC1 are indicated by dots. Refer to the legend of Figure 1 for abbreviation used.

MHC found in larvae and postlarva at 20 dpm. Because two sequences were considerably different from those of MHCs of adult muscles and similar to those of larval and postlarval MHCs from black tiger shrimp, MHCs from postlarvae at 3 and 20 dpm were named pvMHC2P1 (AB759105) and pvMHC2P2 (AB759104), respectively (Fig. 2).

The amino acid identities of larval and postlarval MHCs to MHC1 were 69–70% and those to MHC2 were 93–94% (Table 3).

Phylogenetic Relationships

Figure 3 shows the phylogenetic tree based on the partial amino acid sequences of MHCs from kuruma, black tiger, and Pacific white shrimps in comparison with those of various animals. The tree demonstrated that MHC1 from abdominal muscle formed one clade with MHC3 and MHC4 from pleopod muscle except pvMHC5 from adult Pacific white shrimp pleopod muscle, whereas MHC2 formed another clade with MHCs from larvae and postlarvae. Two MHCs, MHCpa (AB759094) and MHCpb (AB759095), cloned from postlarva of black tiger shrimp in our previous study (Koyama et al., 2012b) were monophyletic with MHC1. Thus we renamed MHCpa and MHCpb to pmMHC1P1 and pmMHC1P2, respectively. pvMHC5 from adult Pacific white shrimp pleopod muscle was monophyletic with American lobster S_2 slow tonic MHC.

DISCUSSION

It has been confirmed that *MHC1* and *MHC2* transcripts are localized in adult abdominal muscle of the three shrimp species by in situ hybridization and Northern blot analysis, whereas both transcripts were not detected in adult pleopod muscle (Koyama et al., 2012a,b). In this study, we cloned *MHC*s encoding C-terminal sequences from adult pleopod muscle of the three shrimps by using a degenerate forward primer and GeneRacer 3′ Primer. Two types of MHC, neither MHC1 nor MHC2, were cloned from kuruma shrimp pleopod muscle and named as mjMHC3 and mjMHC4. Two types of MHCs were also cloned from Pacific white shrimp pleopod muscle and named as pvMHC4 and pvMHC5, whereas one type of MHC was determined from black tiger shrimp pleopod muscle and named as pmMHC3.

Table 3. The amino acid identity of myosin heavy chains from abdominal muscle of adult specimens of black tiger and Pacific white shrimp together with those of their whole larvae and postlarvae (%).

	Abdominal		Larval			Abdominal		Larval	
	pmMHC1	pmMHC2	pmMHC2Z	pmMHC2M	pmMHC2P	pvMHC1	pvMHC2	pvMHC2P1	pvMHC2P2
pmMHC1[a]		71	70	69	70	96	71	69	70
pmMHC2			94	93	94	71	99	93	94
pmMHC2Z				98	99	70	94	95	97
pmMHC2M					98	70	94	95	97
pmMHC2P						70	94	95	98
pvMHC1[a]							72	70	70
pvMHC2								93	94
pvMHC2P1									96

[a]Abbreviations used are: MHC, myosin heavy chain; pm, black tiger shrimp *Penaeus monodon*; pv, Pacific white shrimp *Penaeus vannamei*.

The pleopod muscles from shrimps were oxidative fibers according to NADH-diaphorase staining (Koyama et al., 2012a, b). However, it is unclear whether pleopod muscle was of fast or slow type, because fast-type oxidative fibers have been observed for decapod crustaceans (Silverman and Charlton, '80; Hardy et al., 2010). The phylogenetic tree was constructed based on partial MHC sequences (see Fig. 3). MHC3 and MHC4 except pvMHC5 from pleopod muscle were monophyletic with MHC1. Since MHC1 is of fast type, MHC3 and MHC4 except pvMHC5 are considered to be of fast type. pvMHC5 from Pacific white shrimp pleopod muscle was monophyletic with American lobster S_2 slow tonic MHC, thus pvMHC5 is considered to be of slow type. This is the first report to identify slow-type MHC from above-mentioned three shrimp species. These results indicate that both fast- and slow-type MHCs exist in pleopod muscle of Pacific white shrimp. Slow-type MHCs have not been cloned yet from kuruma and black tiger shrimps, which requires further investigation. In addition, it has not been confirmed whether or not *MHC3* and *MHC4* transcripts are localized in abdominal muscle, which is our next investigation in the future by using in situ hybridization and Northern blot analyses.

Two *MHC*s, *MHC1P1* and *MHC1P2*, were cloned from postlarvae of black tiger shrimp in our previous study, although their days after metamorphosis were not known (Koyama et al., 2012b). We cloned novel larval- and postlarval-type MHCs from black tiger and Pacific white shrimps in this study. Larval- and postlarval-type MHCs were cloned from zoea, mysis, and postlarvae. However, no clone was isolated from black tiger shrimp at nauplius stage. The deduced amino acid sequences of pmMHC2Z from zoea, pmMHC2M from mysis and pmMHC2P from postlarvae of black tiger shrimp were different from those of pmMHC1P1 and pmMHC1P2 cloned in our previous study (Table 4). While pmMHC1P1 and pmMHC1P2 in postlarvae were monophyletic with pmMHC1, other MHCs from various developmental stages of black tiger shrimp, pmMHC2Z, pmMHC2P, and pmMHC2M, were monophyletic with pmMHC2, indicating that MHC1 and MHC2 have respective larval/postlarval-type MHCs. It has been revealed in our previous study that the three shrimps express MHC1 and MHC2 transcripts in flexor abdominal muscle and only MHC2 in extensor abdominal muscle, indicating the functional differences of two MHCs (Koyama et al., 2012a,b). It is interesting to make clear whether or not MHC1 is expressed at a later postlarval stage in abdominal muscle. Alternatively, two larval/postlarval-type MHCs, belonging to MHC1 and MHC2 families, are assumed to be expressed in larval and postlarval stages, which is our next target to study. Larval-type MHCs were also cloned from larvae of Pacific white shrimp. pvMHC2P2 was expressed almost constantly throughout different developmental stages (Table 4), although it is known that vertebrates change the expression of MHC isoforms during their development (Lyons et al., '90; Agbulut et al., 2003; Nihei et al., 2006; Ono et al., 2006, 2010).

Although loop 1 region from the larvae of European lobster *H. gammarus* was cloned (Magnay et al., 2003), the sequences of loop regions were not determined for shrimp larvae and postlarvae in this study. To consider the functional differences among MHCs, it is necessary to clone loop regions of larval and postlarval MHCs and to compare their length and net charges.

In conclusion, novel MHCs were identified from pleopod muscle of the three shrimp species and from larvae and postlarvae of black tiger and Pacific white shrimps. It is notable that slow-type MHC was identified in pleopod muscle of Pacific white shrimp, although other MHCs obtained from pleopod muscle in this study were of fast type. In addition, it was confirmed that *MHC* expression was altered depending on their developmental stages. These new

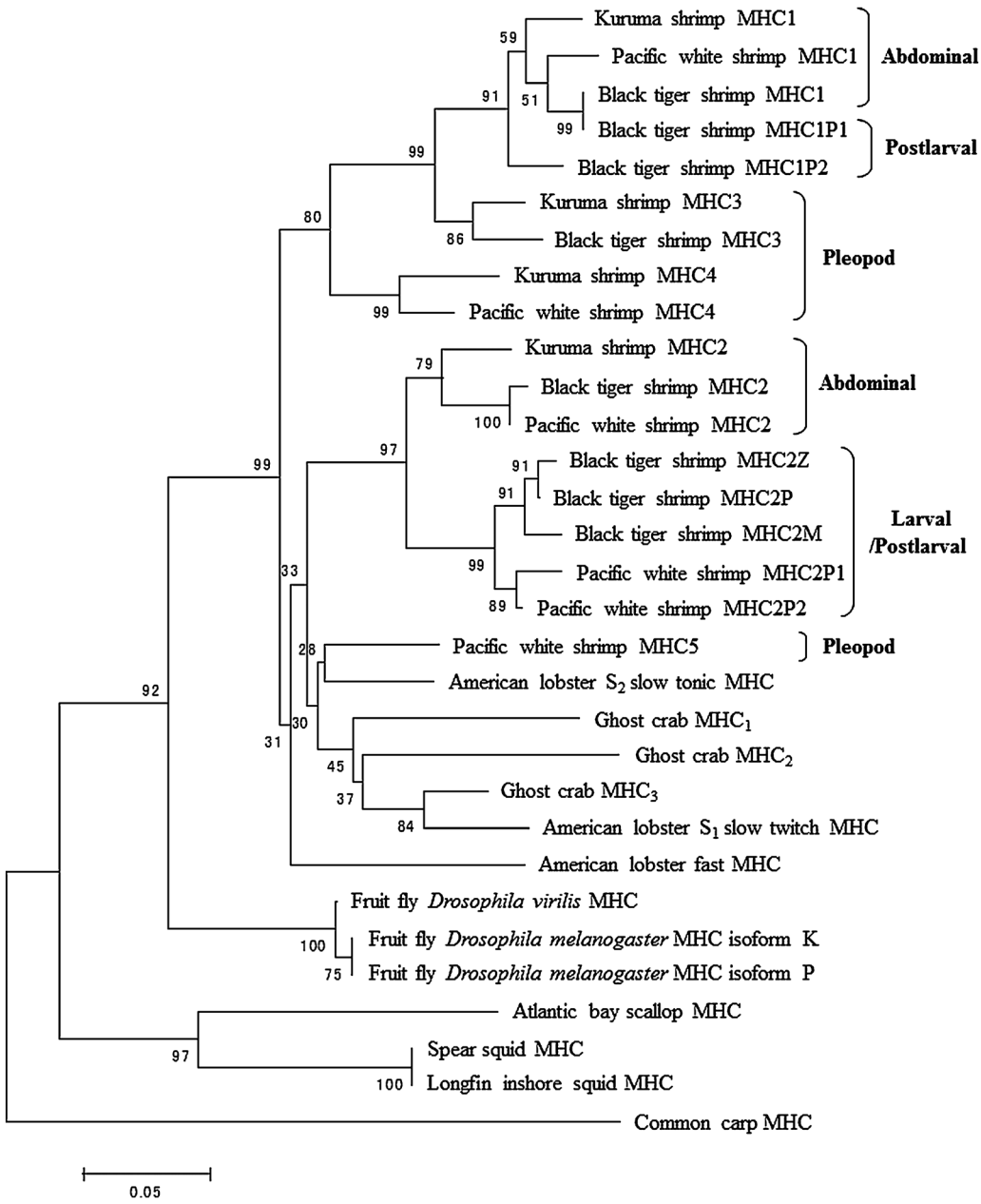

Figure 3. The phylogenetic tree of myosin heavy chains (MHCs) from kuruma, black tiger, and Pacific white shrimps together with those from other species. MHCs cited are: kuruma shrimp *Marsupenaeus japonicus* (MHC1, AB613205; MHC2, AB613206); black tiger shrimp *Penaeus monodon* (MHC1, AB758441; MHC2, AB758442; MHC1P1, AB759094; MHC1P2, AB759095); Pacific white shrimp *P. vannamei* (MHC1, AB758443; MHC2, AB758444); American lobster *Homarus americanus* (fast, U03091; S_1 slow twitch, AY232598; S_2 slow tonic, AY521626); ghost crab *Ocypode quadrata* (MHC_1, DQ534440; MHC_2, DQ534441; MHC_3, EU676338); flies *Drosophila melanogaster* (isoform K, NP724008; isoform P, NP001162992) and *D. virilis* (XM002051957); scallop *Argopecten irradians* (X55714), squids *Loligo pealei* (AAC24207) and *L. bleekeri* (ACD68201); common carp *Cyprinus carpio* (D89990).

Table 4. The expression profiles of myosin heavy chains from black tiger and Pacific white shrimps at various developmental stages.

			Postlarva			
Species	Zoea	Mysis	At 3 dpm	At 20 dpm	Unknown	Adult
Black tiger shrimp	pmMHC2Z[a]	pmMHC2M			pmMHC1P1	pmMHC1
					pmMHC1P2	pmMHC2
					pmMHC2P	pmMHC3
						pmMHC4
Pacific white shrimp	pvMHC2P2[a]	pvMHC2P2	pvMHC2P1	pvMHC2P2		pvMHC1
						pvMHC2
						pvMHC4
						pvMHC5

[a]Abbreviations used are: MHC, myosin heavy chain; pm, black tiger shrimp *Penaeus monodon*; pv, Pacific white shrimp *Penaeus vannamei*.

findings will help understand the physiology and biochemistry of crustacean muscles.

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