

Identification and Analysis of Muscle-Related Protein Isoforms Expressed in the White Muscle of the Mandarin Fish (*Siniperca chuatsi*)

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Abstract To identify muscle-related protein isoforms expressed in the white muscle of the mandarin fish *Siniperca chuatsi*, we analyzed 5,063 high-quality expressed sequence tags (ESTs) from white muscle cDNA library and predicted the integrity of the clusters annotated to these genes and the physiochemical properties of the putative polypeptides with full length. Up to about 33% of total ESTs were annotated to muscle-related proteins: myosin, actin, tropomyosin/troponin complex, parvalbumin, and Sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCa). Thirty-two isoforms were identified and more than one isoform existed in each of these proteins. Among these isoforms, 14 putative polypeptides were with full length. In addition, about 2% of total ESTs

were significantly homologous to “glue” molecules such as alpha-actinins, myosin-binding proteins, myomesin, tropomodulin, cofilin, profilin, twinfilins, coronin-1, and nebulin, which were required for the integrity and maintenance of the muscle sarcomere. The results demonstrated that multiple isoforms of major muscle-related proteins were expressed in *S. chuatsi* white muscle. The analysis on these isoforms and other proteins sequences will greatly aid our systematic understanding of the high flexibility of mandarin fish white muscle at molecular level and expand the utility of fish systems as models for the muscle genetic control and function.

Keywords Mandarin fish · ESTs · White muscle · Muscle protein · Isoforms

Guoqing Zhang and Wuying Chu contributed equally to this work.

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Introduction

Fish skeletal muscles consist of two spatially separated fibers. White, fast-twitch muscles make up the bulk of the body of fish, whereas red, while slow-twitch fibers are found in a narrow midlateral band immediately under the skin (McGuigan et al. 2004). Many proteins are specifically expressed in muscle tissues, and these mainly include structure and contractile proteins (e.g., myosin, actins, and troponin) as well as soluble muscle proteins and muscle-specific regulating factors (Rodgers et al. 1987; Xu et al. 2000; Grimaldi et al. 2004). Various swimming gaits of fish depend on the different physiological properties and recruitment of the various myotomal muscle fiber types. White, fast-twitch muscles are recruited for faster swimming speeds (Maddock et al. 1994; Coughlin et al. 2005), whereas red, slow-twitch muscle for low undulatory swimming

speeds (Rome et al. 1984). Variances in the physiological and biochemical properties of different muscle fiber types, responsible for the diverse contraction and relaxation of muscles, are attributable to different isoforms of muscle proteins in the muscle tissues (Berchtold et al. 2000).

The mandarin fish, popular in northeastern Asian countries, is carnivorous and naturally lives in many eastern Asian areas due to its broad temperature tolerance range. When the temperature rises above 5°C, mandarin fish begins to prey moving targets swiftly. The swiftness of the mandarin fish under broad temperature range supposedly results from different isoforms of muscle proteins, especially those in white muscles that make up the bulk of the mandarin fish's body (Rome et al. 1984; Maddock et al. 1994; Berchtold et al. 2000; Coughlin et al. 2005). Even though much work on muscle-related proteins sequences or genes is available (Guo et al. 1994; Imai et al. 1997; Hirayama et al. 1998; Xu et al. 2000; Weaver et al. 2001; Bryson-Richardson et al. 2005; Fukushima et al. 2009; Wang et al. 2008b), more detail sequence information on muscle-related proteins appears to be important for the better understanding of mandarin fish's broad adaptive capacity to diverse conditions.

EST sequencing is one of the most efficient means for gene discovery and molecular marker development, and it has been widely applied in fish biology to discover novel, uniquely expressed genes, and characterize expression profiles of distinctly expressed genes under different stimuli (Reusch et al. 2008; Wynne et al. 2008), in various organs (Li et al. 2007) and in genetic mapping and comparative genome analysis by identifying polymorphic DNA markers, such as microsatellites (Kucuktas et al. 2009) and single nucleotide polymorphisms (Sarropoulou et al. 2008; von Schalburg et al. 2008; Wang et al. 2008a; Kucuktas et al. 2009). Compared with the conventional approaches of identifying protein isoforms, such as two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and immunological blotting, EST sequencing can quickly and inexpensively provide the expression profiles and sequences of different muscle-related protein isoforms on large scale (Li et al. 2007). The primary goal of this study is to identify the major isoforms of muscle-related protein expressed in the mandarin fish white muscle and compare their physiological and biochemical properties with others. Therefore, we analyzed 5,063 high-quality ESTs from the mandarin fish *S. chuatsi* white muscle cDNA library and predicted the integrity and the preliminary properties of the derived peptides from ESTs annotated to muscle-related genes. This first comprehensive identification of the main isoforms of muscle-related proteins should greatly deepen our understanding of the highly functional plasticity of fish muscle at molecule level, in response to varying functional demands and environmental changes.

Materials and Methods

cDNA Library Construction and EST Sequencing

Total RNA was extracted from the *S. chuatsi* muscle samples using Unizol reagent (Biostar, Shanghai, China). Poly(A)+mRNA was isolated from total RNA using Oligotex mRNA mini kit (Qiagen). Then poly(T)-primed cDNA was synthesized and directionally inserted into the pBluescript_II_SK(+) vector. DNA was transformed by electroporation into the *Escherichia coli* strain DH10B. The unnormalized library contained an average insert size of 1.7 kb. Using DYEnamic ET Terminator (GE Healthcare) with a T3 primer (5'-AATTAACCTCACTAAAG-3'), 5,456 single-pass sequencing reactions from 5' end of each cDNA clones were carried out. The sequencing was performed on a 3730XL DNA analyzer (Applied Biosystems, Foster City, CA).

Data Processing, Cluster Assembly, and Bioinformatics Analysis

We processed the chromatogram files sequencing from 5' end as raw data for base-calling and quality assessing by Phred software (Phred-Phrap-Consed package). The low-quality sequences were trimmed off with Q13 (95% accuracy), and the vector sequences were screened with CROSS_MATCH program. All high-quality ESTs (sequences more than 100 bp after quality and vector trimming) were assembled by CAP3 software using default settings (overlap length cut-off >30 and overlap percent identity cut-off >75) (Huang and Madan 1999). Assembled clusters were manually revised by Consed software (Gordon et al. 1998) and then searched against UniProtKB/Swiss-Prot protein database in by BLASTX with *e* values 1e-5.

Clusters were considered as full length if they contained a complete ORF (open-reading frame), identified by BLASTX and start and stop codons as well as poly(A) tail (Min et al. 2005b). For skeletal muscle-specific or enriched transcripts without complete coding regions, appropriate clones were sequenced from 3' ends again. Clones were subjected to further sequencing by primer walking to get the full insert of the clones after both 5' and 3' EST sequencing. All the sequences including 5' and 3' ESTs and extended sequences were processed and were finally assembled again using CAP3 as above.

Protein Analysis

Putative peptides were conceptually translated with OrfPredictor (Min et al. 2005a) and verified by searching against the protein database with BLSATP. Multiple sequence alignments were carried out using MUSCLE

(Edgar 2004) or CLUSTAL_X (Larkin et al. 2007). The physiochemical properties of proteins were analyzed using ExPASy webserver.

Results and Discussions

Overview of ESTs from the *S. chuatsi* Skeletal Muscle cDNA Library

We sequenced at the 5'-terminus of each insert on 5,456 randomly selected clones from a cDNA library of the mandarin fish white muscle. After discarding the poor-quality and vector sequences, 5,191 readable sequences were produced, and 5,063 sequences were longer than 100 base pairs. The average length of these ESTs was 587 bp (Fig. 1). All the ESTs were deposited to dbEST [GenBank: GR473858–GR479048]. Subsequently, all high-quality 5' ESTs were assembled into 1,691 clusters: 284 contigs (contigs refer to clusters consisting of more than one EST) and 1,407 singletons (singletons refer to clusters consisting of just one EST).

After annotating the ESTs, clusters significantly matched to myofibrillar proteins, including myosin, actin, tropomyosin, and troponin complex, and they were abundant accounting about 24% of total ESTs (Fig. 2). More than one isoform existed in each of these myofibrillar proteins, and up to 25 isoforms were identified in these myofibrillar proteins, and eight of them were with complete ORFs (Table 1), as detailed later. Transcripts coding for proteins related to calcium binding/transporting such as parvalbumin and sarco/endoplasmic reticulum (SR) calcium transport ATPase (SERCa) were also rich, which accounted about 9% of total ESTs. All five isoforms identified in parvalbumin were with complete ORFs. Two isoforms were identified in SERCa, and one of them was with complete ORF (Table 1). In addition, other calcium-binding proteins such as calsequestrin, which helps hold

calcium in the cisterna of the sarcoplasmic (Wang et al. 1998), were also identified.

The integrity and maintenance of the muscle sarcomere require a complex interplay of so-called “glue” molecules that bind myosin/actin and link various filaments together or anchor filaments to membrane. Transcripts homologous to such “glue” molecules, up to 2% of total ESTs, also existed in *S. chuatsi* (Table 2). BLASTX searches against Swiss-Prot showed that 13 ESTs significantly matched to myosin-binding proteins, 3 matches to myomesin (another myosin-binding protein), and 10 ESTs homologous to alpha-actinin that cross-links actin filaments and titin molecules (Nwe et al. 1999; Atkinson et al. 2000). Other proteins regulating the length of actin filament were also identified, such as myozenin, tropomodulin, cofilin, profilin, twinfilins, coronin-1, and nebulin (Amali et al. 2004; Campinho et al. 2005).

Identification and Analysis of Transcriptional Isoforms of the Major Myofibrillar Proteins

Myosin Structure

Conventional myosin contains two heavy chains (MYH) and four light chains (MYL) (Rodgers et al. 1987). The C-terminal of each MYH, which defines the maximal shortening velocity of muscle (Moss et al. 1996), folds into α -helical coiled-coil structure and joins two MYHs together (Weeds and McLachlan 1974). In contrast, its N-terminal is composed of two regions, the head domain that binds the filamentous actin and hydrolyzes ATP to generate force to “walk” along the filament, and the neck domain that acts as a linker binding two myosin light chains together and as a lever arm for transducing force generated by the head domain (Ruegg et al. 2002). Prior to this study, there were five myosin sequences of mandarin fish *S. knerii* skeletal muscle MYLs (myosin light chains) deposited in GenBank: four myosin light chain sequences (Zhang et al., unpublished): MYL1_SKr (gb|ACJ47229.1), MYL3_SKr (gb|ACJ12595.1), MYL3b_SKr (gb|ACJ47231.1), and MYL2_SKr (gb|ACJ47230.1), and one myosin heavy chain sequence MYH_SKr (gb|ABO31103.1) (Zhang et al. 2009a, b).

Myosin Light Chain

Five clusters were homologous to myosin light chains or polypeptides (MYLs) (Table 1). Two clusters contained complete open reading frames. One consisting of 37 ESTs that encoded a polypeptide of 192 amino acids (aa) and was designated MYL1_SC, which had the same sequence as MYL1_SKr. Another of 257 ESTs, designated MYL3_SC, encoded a polypeptide of 150 amino acids and was also

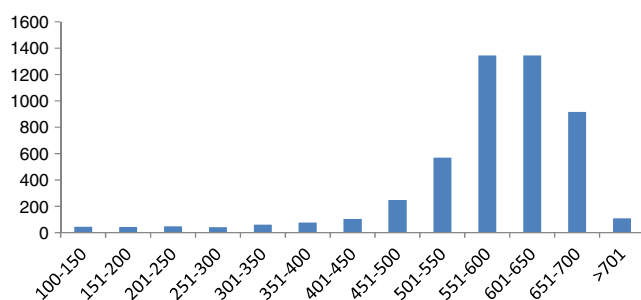
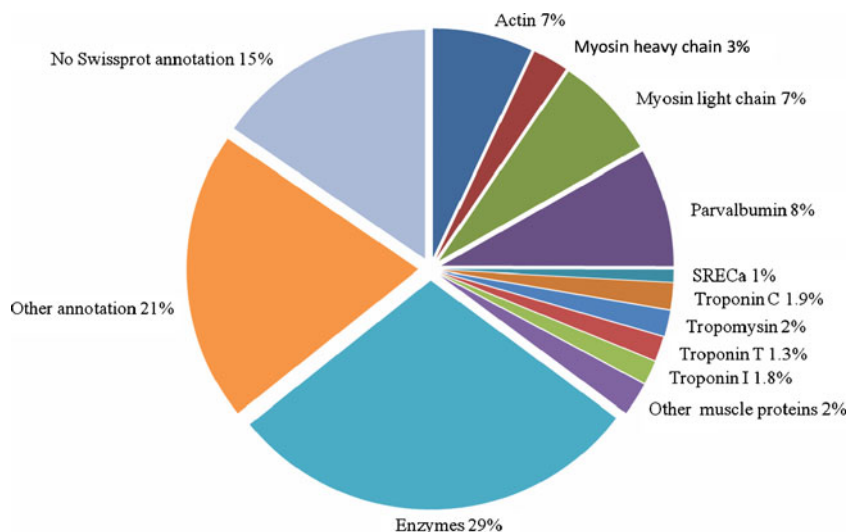


Fig. 1 Length distribution of 5' ESTs from the *Siniperca chuatsi* skeletal muscle cDNA library. A total of 5,063 high quality 5' ESTs were analyzed in the current study. Abscissa is the sequence length in 50-bp intervals, while the ordinate is the number of ESTs

Fig. 2 Functional annotation of ESTs annotated to muscle-related genes. The relative proportion of different types of transcripts: myosin (further divided into light chain, and heavy chain), actin, troponin (further divided into troponin C, I, and T), tropomyosin, parvalbumin, SERCa, other muscle proteins, other Swiss-Prot annotation, and no Swiss-Prot annotation



identical to MYL3_SKr. The rest 3 clusters contained incomplete open reading frames. Thus, there appears to be at least five MYL isoforms in the mandarin fish skeletal muscles. Previous studies demonstrated that two essential light chain isoforms (MYL1 and MYL3) in birds and mammals were produced from a single gene by alternative splicing (Periasamy et al. 1984). In this study, MYL1_SC and MYL3_SC differed in many variances that scattered in the whole sequence and appeared to be produced by two different genes, consistent with other findings on MYL1 and MYL3 in other fishes (Dalla Libera et al. 1991). The MYL3_SC isoform, the major isoform, was 150 aa in length, which would lead to a polypeptide of 16.65 kDa with a theoretical isoelectric point (pI) of 4.42. As with other vertebrates, this MYL lacked the Pro-Ala-rich N-terminal extension of the long vertebrate essential light chain isoform. Comparative sequence analysis revealed that MYL3_SC shared 95% identity with MLE3_MUGCA, myosin light chain 3 of *Liza ramada* *Liza ramado* skeletal muscle isoform (P82160).

MYL1_SC isoform, the minor isoform, was 192 amino acids in length resulting in a polypeptide of 20.75 kDa with a theoretical pI of 4.62. In comparison with MYL3_SC, MYL1_SC isoform had an N-terminal extension of 41 aa and showed 94% identity with MLE1_MUGCA, myosin light chain 1 of *Liza ramada* *L. ramado* skeletal muscle isoform (P82159.1). This N-terminal extension consisted of a repetitive Ala-Pro-rich segment (20 aa) and a highly charged interactive “sticky” element (residues 1–15) at the most N-terminus (Hayashibara and Miyanishi 1994; Timson et al. 1999), which was bound to a cluster of C-terminal acidic residues 360–364 of actin (Trayer et al. 1987; Hirayama et al. 2000).

It was observed that the discrepant amino acids were scattered in the inter regions of two EF-hand region; the conserved amino acids were concentrated in EF-hand

regions, and EF-hand II was more conserved than EF-hand I (Fig. 1A, Supplemental File). Essential light chain belongs to the EF-hand family of calcium-binding proteins. Therefore, EF-hand II was supposed to be the main calcium-binding sites, at least in MYL1_SC, because it was highly conservative and remote from the repetitive Ala-Pro-rich segment in MYL1_SC (Huriaux and Focant 1977).

Myosin Heavy Chain

The conventional myosin heavy chain (MYH) generally has a MW of about 220 kDa encoding more than 1,900 aa. It can be subdivided into three fragments: S1 (or subfragment 1, the head), S2 (subfragment 2, the proximal one third of the tail), and light meromyosin (LMM, the distal two thirds of the tail) (Kikuchi et al. 1999). Six clusters were annotated to different parts of the common carp *Cyprinus carpio* skeletal muscle isoform of myosin heavy chain (MYSS_CYPCA: Q90339) (Fig. 3). Three clusters were homologous to the head of MYSS_CYPCA and other three clusters to the tail of MYSS_CYPCA. So far, one cluster encoding complete globular head region and another complete LMM fragment were obtained, although none of them were complete MYH.

Three clusters matched the head region of MYH. The largest was composed of 46 ESTs and designated as MYHHead1_SC. It deduced a 932 aa of MYSS_CYPCA and possessed the start codon and 5' UTR. Another cluster deduced a 192 aa polypeptide and was designated as MYHHead2_SC. The other deduced a 433 aa polypeptide and was designated as MYHHead3_SC. All these three clusters showed highest homology to MYSS_CYPCA, with 86% identity for MYHHead1_SC, 90% for MYHHead2_SC, and 87% for MYHHead3_SC (Table 1).

In the multi-sequences alignment, MYHHead1_SC was with the same sequence as the MYHHead_SKr (Zhang et al.,

Table 1 Transcriptional isoforms of mandarin fish muscle filament proteins

Protein	Abbreviation	CDS	Length of propeptide	Clones	pI	Mw (Da)	Best hit	% identity	Organism of best hit
MYL	MYL1_SC	Complete	192	37	4.62	20,752.5	MLE1_MUGCA	94	Liza ramada
	MYL2_SC	Partial, lack C-terminal	120	7	–	–	MLRT_RABIT	83	Rabbit
	MYL3_SC	Complete	150	257	4.42	16,652.82	MLE3_MUGCA	95	Liza ramada
	MYL4_SC	Partial, lack N-terminal	116	5	–	–	MYL6_RAT	82	Norway rat
	MYL5_SC	Partial, with N-terminal	99	8	–	–	MYL9_PONAB	92	Sumatran orangutan
MYH	MYHhead1_SC	Partial, with N-terminal	932	46	–	–	MYSS_CYPCA	86	Common carp
	MYHhead2_SC	Partial	192	2	–	–	MYSS_CYPCA	90	Common carp
	MYHhead3_SC	Partial	433	1	–	–	MYSS_CYPCA	87	Common carp
	MYHtail1_SC	Partial, with C-terminal	772	52	–	–	MYSS_CYPCA	84	Common carp
	MYHtail2_SC	Partial	606	4	–	–	MYSS_CYPCA	83	Common carp
	MYHtail3_SC	Partial	190	2	–	–	MYSS_CYPCA	78	Common carp
	ACT1_SC	Complete	377	345	5.22	41,944.89	ACTS_OREMO	100	Mozambique tilapia
	ACT2_SC	Partial, with N-terminal	190	3	–	–	ACTB_SALSA	99	Atlantic salmon
	ACT3_SC	Partial, with N-terminal	107	2	–	–	ACTB_XENTR	100	Western clawed frog
	TPM1_SC	Complete	284	75	4.69	32,683.62	TPM1_LIZAU	94	Golden gray mullet
Tropomyosin	TPM2_SC	Partial, lack N-terminal	150	19	–	–	TPM1_LIZAU	97	Golden gray mullet
	TPM3_SC	Partial	132	1	–	–	TPM3_MOUSE	83	House mouse
	TNNT1_SC	Complete	228	69	4.64	21,775.22	TNNT3_COTJA	49	Japanese quail
Troponin T	TNNT2_SC	partial, with N-terminal	188	2	–	–	TNNT3_RAT	48	Norway rat
	TNNT3_SC	Complete	171	81	9.27	19,243.47	TNNI2_COTJA	54	Japanese quail
Troponin I	TNNI1_SC	Partial, lack N-terminal	154	2	–	–	TNNI2_COTJA	50	Japanese quail
	TNNI2_SC	Partial, lack N-terminal	131	2	–	–	TNNI2_COTJA	57	Japanese quail
	TNNI3_SC	Partial, lack C-terminal	175	1	–	–	TNNI1_HUMAN	72	Human
	TNNI4_SC	Complete	160	84	3.96	18,188.1	TNNC2_ANGAN	79	European eel
Troponin C	TNNC1_SC	Complete	161	2	3.91	18,210.01	TNNC3_ANGAN	86	European eel
	TNNC2_SC	Complete	109	235	4.6	11,589.16	PRVA_CYPCA	75	Common carp
Parvalbumin	PRV1_SC	Complete	109	157	4.65	11,466.9	PRVB_SCOJP	85	Chub mackerel
	PRV2_SC	Complete	109	15	4.46	11,495.91	PRVB_SCOJP	80	Chub mackerel
	PRV3_SC	Complete	109	1	4.6	11,617.22	PRVA_CYPCA	74	Common carp
	PRV4_SC	Complete	108	1	4.58	11,957.62	PRV7_DANRE	67	Zebrafish
	PRV5_SC	Complete	957	30	5.09	10,4827.12	AT2A1_MAKNI	90	Atlantic blue marlin
SERCα	SERCα1_SC	Complete	353	9	–	–	AT2A2_MAKNI	82	Atlantic blue marlin
	SERCα2_SC	Partial, with N-terminal	–	–	–	–	–	–	–

The abbreviations of mandarin fish muscle filament protein transcriptional isoforms, the completeness of their coding sequences (CDS), their lengths of the putatively deduced polypeptides, the number of clones comprising the isoforms, the predicted pI and Mw, and their best BLAST hits in Swiss-Prot database as well as the identity and the common names of the organism of best hits are shown

Table 2 Functional distribution of the 5' ESTs annotated to other muscle-related genes based on Swiss-Prot matches by BLASTX

Query name	EST counts	Length	Accession No.	Identity	Description
Contig65	3	715	sp P20111 ACTN2_CHICK	86	Alpha-actinin-2
Contig187	6	1,532	sp Q0III9 ACTN3_BOVIN	88	Alpha-actinin-3
gy_0035_D06.ab1	1	553	sp Q0III9 ACTN3_BOVIN	76	Alpha-actinin-3
gy_0011_A02.ab1	1	627	sp P31231 CASQ1_RANES	50	Calsequestrin-1
Contig209	2	798	sp Q6B7M7 COF1_SHEEP	60	Cofilin-1
Contig84	2	644	sp Q5G6V9 COF2_PIG	75	Cofilin-2
gy_0056_G02.ab1	1	667	sp Q5G6V9 COF2_PIG	93	Cofilin-2
gy_0042_C10.ab1	1	521	sp Q9XS70 COR1B_RABIT	57	Coronin-1B
Contig170	5	1,210	sp Q9D119 DYSII_MOUSE	68	Dysferlin-interacting protein 1
Contig255	8	1,322	sp Q9D119 DYSII_MOUSE	73	Dysferlin-interacting protein 1
gy_0057_F05.ab1	1	619	sp Q9D2N4 DTNA_MOUSE	83	Dystrobrevin alpha
gy_0033_C02.ab1	1	486	sp Q14315 FLNC_HUMAN	87	Filamin-C
Contig94	2	925	sp P18520 ION3_CARAU	81	Intermediate filament protein ON3
gy_0019_F09.ab1	1	627	sp Q99JW4 LIMS1_MOUSE	84	LIM and senescent cell antigen-like-containing domain protein 1
gy_0028_B10.ab1	1	629	sp Q8QFP8 LIMK1_CHICK	72	LIM domain kinase 1
gy_0032_A08.ab1	1	548	sp P53668 LIMK1_MOUSE	83	LIM domain kinase 1
gy_0008_C02.ab1	1	599	sp P52179 MYOM1_HUMAN	60	Myomesin-1
gy_0006_C07.ab1	1	710	sp Q62234 MYOM1_MOUSE	66	Myomesin-1
gy_0038_G04.ab1	1	600	sp P54296 MYOM2_HUMAN	50	Myomesin-2
gy_0007_F08.ab1	1	675	sp Q5XKE0 MYPC2_MOUSE	64	Myosin-binding protein C, fast-type
Contig158	7	832	sp Q05623 MYBPH_CHICK	53	Myosin-binding protein H
Contig224	5	1,214	sp Q05623 MYBPH_CHICK	63	Myosin-binding protein H
gy_0021_F01.ab1	1	681	sp Q27966 MYO1C_BOVIN	63	Myosin-Ic
gy_0049_F01.ab1	1	281	sp Q9UBF9 MYOTI_HUMAN	56	Myotilin
gy_0011_H12.ab1	1	613	sp Q9Y217 MTMR6_HUMAN	72	Myotubularin-related protein 6
gy_0013_A12.ab1	1	557	sp Q9Y216 MTMR7_HUMAN	62	Myotubularin-related protein 7
Contig101	5	1,681	sp Q4PS85 MYOZ1_PIG	57	Myozenin-1
Contig184	2	794	sp P20929 NEBU_HUMAN	57	Nebulin
gy_0037_B06.ab1	1	674	sp P20929 NEBU_HUMAN	45	Nebulin
gy_0048_C05.ab1	1	667	sp Q86VF7 NRAP_HUMAN	56	Nebulin-related-anchoring protein
gy_0041_E07.ab1	1	646	sp Q62920 PDLI5_RAT	62	PDZ and LIM domain protein 5
Contig247	9	1,097	sp Q3SX40 PDLI7_BOVIN	45	PDZ and LIM domain protein 7
gy_0008_E05.ab1	1	611	sp Q6P7E4 PDLI7_DANRE	63	PDZ and LIM domain protein 7
gy_0014_H04.ab1	1	701	sp Q15149 PLEC1_HUMAN	69	Plectin-1
gy_0021_C11.ab1	1	484	sp Q9QXS1 PLEC1_MOUSE	55	Plectin-1
gy_0045_H06.ab1	1	646	sp Q32PB1 PROF3_BOVIN	35	Profilin-3
Contig174	6	1,359	sp Q6T8D8 TELT_BOVIN	41	Telethonin
gy_0048_A08.ab1	1	650	sp Q8CGB6 TENC1_MOUSE	41	Tensin-like C1 domain-containing phosphatase
Contig58	2	834	sp Q8WZ42 TITIN_HUMAN	57	Titin
gy_0025_C09.ab1	1	665	sp Q8WZ42 TITIN_HUMAN	65	Titin
gy_0026_D03.ab1	1	608	sp Q8WZ42 TITIN_HUMAN	74	Titin
gy_0007_G12.ab1	1	635	sp A2ASS6 TITIN_MOUSE	58	Titin
gy_0008_H12.ab1	1	657	sp A2ASS6 TITIN_MOUSE	67	Titin
gy_0011_G11.ab1	1	641	sp A2ASS6 TITIN_MOUSE	64	Titin
gy_0039_D09.ab1	1	595	sp A2ASS6 TITIN_MOUSE	63	Titin
Contig64	5	1,479	sp A0JNC0 TMOD1_BOVIN	61	Tropomodulin-1
Contig114	2	763	sp Q6P9T8 TBB2C_RAT	87	Tubulin beta-2C chain
gy_0024_B12.ab1	1	662	sp Q6GMH3 TWF2_DANRE	80	Twinfilin-2

The clusters annotated to other muscle-related genes, the numbers of ESTs, their length, and the accession no. of their best BLAST hits in Swiss-Prot database as well as the identity and the description of the best hits are shown

unpublished) and MYHHead3_SC was more different to MYHHead1_SC from MYHHead2_SC to MYHHead1_SC. The different amino acids were scarcely located in the ATP-binding and actin-binding domain (Fig. 2, Supplemental File), which produce force and transduces it along the filament (Ruegg et al. 2002). They were concentrated in the less-conserved regions such as the Loop I region, which was near the nucleotide-binding pocket and likely to tune the rate constant for ADP release (Goodson et al. 1999).

Three clusters significantly matched different tail regions of MYSS_CYPKA. The most abundant transcript was composed of 52 ESTs and designated as MYHTail1_SC. It deduced a 772 aa polypeptide sharing 84% identity with the C-terminal of MYSS_CYPKA and possessed the stop codon and 3' UTR. Other two clusters encoded the polypeptides of 606 aa and 190 aa long, and they were designated as MYHTail2_SC and MYHTail3_SC, respectively. MYHTail2_SC shared 83% identity with the counterparts of MYSS_CYPKA and MYHTail2_SC 78% identity.

In alignment with MYHTail_SKr, MYHTail1_SC differed six amino acids, and two of them (T507R and N509Q) located in the S2 region, the remaining four existed in the LMM region. MYHTail2_SC had the same replacement in the S2 region of MYHTail_SKr and the third different amino acid (A792T) occurred in LMM region (Fig. 3, Supplement file). With respect to MYHTail3_SC, there were apparent differences in the counterparts of other isoforms (Fig. 4, Supplement File). These differences in LMM region over mandarin fish MYH isoforms may be responsible for its thermo-stability as indicated by other studies (Kakinuma et al. 2000a).

Actin

Actins are highly conserved proteins that are involved in various types of cell motility and ubiquitously expressed in all eukaryotic cells. Three main groups of actins isoforms (alpha, beta, and gamma) exist in vertebrates. The alpha actin exists in muscle tissues and is a major constituent of the contractile apparatus, while the beta and gamma actins coexist in most cell types as components of the cytoskeleton and as mediators of internal cell motility (Venkatesh et al. 1996). There were three clusters significantly matching to actins. The major cluster, designated as ACT1_SC, included 345 ESTs and possessed the start codon, 5' and 3' UTR. ACT1_SC encoded a 377 aa-long polypeptide, which was identical to actin, alpha skeletal muscle of Mozambique tilapia *Oreochromis mossambicus* (ACTS_OREMO: P68264). The cluster of three ESTs, designated as ACT2_SC, deduced a polypeptide of 190 aa sharing 99% identity with the N-terminal of Atlantic salmon *Salmo salar* cytoplasmic actin (ACTB_SALSA: O4216). The cluster of two ESTs deduced the polypeptide identical to a 107 aa-long

region in the C-terminal of western clawed frog *Xenopus (Silurana) tropicalis* cytoplasmic actin 1 (ACTB_XENTR: Q6NVA9) and was designated as ACT2_SC. The results suggested that in *S. chuatsi* white muscle, ACT1_SC is the muscle isoform, and ACT2_SC and ACT3_SC are cytoplasmic isoforms.

The binding sites of ATP (Ser16, Asp156, His163, Glu216, Gly304, and Lys338), gelsolin (Tyr145, Gly148, Glu169, Gly344, Ile347, Leu348, Leu351, Ser352, and Thr353), and profilin (Tyr168, Glu169, Tyr171, Leu173, His175, Asp288, Asp290, Thr353, Glu363, Arg374, and Lys375) to actin were identical in both of the mandarin fish actin isoforms compared with other species (Fig. 5, Supplement File). Profilin enhances actin filament growth, while gelsolin severs actin filament (Okano et al. 1998; Witke et al. 1998). These conserved positions were essential for the dynamics of actin filament.

Tropomyosin/Troponin Complex

Tropomyosin/troponin complex play a central role in the calcium-dependent regulation of vertebrate striated muscle contraction (Kakinuma et al. 2000b; Paul et al. 2009). In striated muscle cells, tropomyosin wraps itself around actin filaments and interacts with troponin through troponin T subunit. Troponin consists of three components: troponin I, which inhibits ATPase activity of actomyosin; troponin T, which contains the binding site for tropomyosin; and troponin C, which binds calcium to abolish the inhibitory action of troponin on actin filaments (Murakami et al. 2007). Different isoforms of each component in tropomyosin/troponin complex can modulate sarcomeric performance by either increasing or decreasing Ca^{2+} sensitivity that regulates muscle contraction and relaxation (Gillis et al. 2007).

Tropomyosin

Tropomyosin (TPM) widely distributes in eukaryotes and associates with actin. Existence of tropomyosin isoforms provides a powerful mechanism to accommodate the diverse functions associated with actin filaments in many cell types. In vertebrates, four genes encoded more than 20 tropomyosin isoforms (Johnson et al. 1967). Three clusters significantly matched to Tropomyosin alpha-1 of golden gray mullet (*Liza aurata*) (TPM1_LIZAU: P84335) were identified. One consisting of 75 ESTs contained a complete open-reading frame coding for a 284 aa-long polypeptide and was designated as the major tropomyosin isoform, TPM1_SC (Zhang et al., unpublished data). It was with theoretical pI of 4.69 with molecular weight of 32.68 kDa (Table 1). Another of 19 ESTs, designated as TPM2_SC, encoded the C-terminal 144 amino acid residues region. There was one difference located in the 284th position

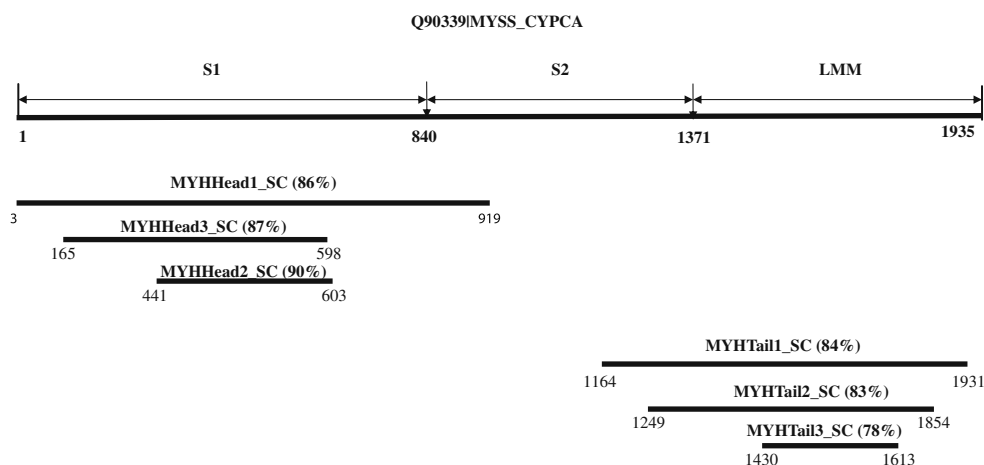


Fig. 3 Schematic of the mandarin fish myosin heavy chain fragments. The mandarin fish myosin heavy chain fragments obtained from EST assembly are placed immediately below the relevant portions of the linear map of *Cyprinus carpio* fast skeletal myosin heavy chain. The numbers under each myosin heavy chain fragment indicate the locations of the start and end positions of its best match in *Cyprinus carpio* fast

skeletal myosin heavy chain. The identity between each myosin II heavy chain fragment and *Cyprinus carpio* fast skeletal myosin heavy chain is shown in parentheses. Abbreviations: Q90339|MYSS_CYPCA, *Cyprinus carpio* fast skeletal myosin heavy chain; S1, myosin subfragment 1; S2, myosin subfragment 2; LMM, L-meromyosin

between TPM1_SC and TPM2_SC. The other cluster of one EST deduced a 132 aa-long polypeptide designated as TPM3_SC. Comparison of putatively deduced polypeptides of TPM1_SC and TPM3_SC revealed that the 12 differences scattered over the region from 135 to 199 aa (Fig. 6, Supplement File). This suggested that the TPM1_SC and TPM3_SC might be produced by two different genes, and there are at least three tropomyosin isoforms in the *S. chuatsi* skeletal muscle.

Troponin T

Two clusters were annotated to troponin T homologues. The most abundant cluster consisted of 69 ESTs and contained a complete open-reading frame for 228 amino acid residues, designated as TNNT1_SC. It showed 49% identity with troponin T of Japanese quail *Coturnix coturnix japonica* (TNNT3_COTJA: P06398). Another cluster of two ESTs, designated as TNNT2_SC, encoded a peptide of 188-long amino acids lacking the C-terminal. TNNT2_SC shared 48% identity with troponin T of Norway rat *Rattus norvegicus* (TNNT3_RAT: P09739) (Table 1). In *S. chuatsi* fast skeletal muscle, the major isoform TNNT1_SC was less conserved than troponin C and troponin I. It showed the highest homology (49% identity) to troponin T in Japanese quail *C. coturnix japonica*, 48% identity to Norway rat *R. norvegicus* and human *Homo sapiens*, 47% identity to house mouse *Mus musculus* and chicken *Gallus gallus*.

In alignment with troponins from other vertebrates, the N-terminal region was highly variable due to the Glu-rich

insertion. The major isoform TNNT1_SC lacked the Glu-rich (23 Es in sum) segment in other vertebrates. In comparison with TNNT1_SC, the minor isoform TNNT2_SC had the Glu-rich insertion of 51 amino acids in length and up to 16 Pro residues, which is aliphatic, has cyclic structure, and plays an important role of stabilizing the protein structure, occurred in this insertion (Fig. 7, Supplement File). The structural alterations of highly variable N-terminal region, caused by its variances of amino acid sequences, length, and charge, affected the binding affinity to troponin I, troponin C and tropomyosin (Wang and Jin 1998; Jin et al. 2000; Jin and Root 2000), and Ca^{2+} activation of ATPase activity (Biesiadecki and Jin 2002). Besides the highly variable N-terminal region, there was one difference between these two troponin T in *S. chuatsi* fast skeletal muscle isoforms in the conserved central region, which contained a second tropomyosin-binding site (Johnson et al. 1967). These differences were supposed to modulate the muscle contractility by affecting the interacting of troponin T with troponin C, troponin I, and tropomyosin directly. The influences of these differences over troponin T isoforms on the contractility of mandarin fish muscle needed further investigation.

Troponin I

Four clusters were homologous to troponin I in *S. chuatsi*. The largest consisted of 81 ESTs and contained a complete ORF encoding a polypeptide of 171 aa in length. Each of the other two clusters consisted of two ESTs. They yielded polypeptides, lacking N-terminal, of 154 and 131 amino

acids in length, respectively. The other cluster of one clone encoded another 175 aa-long polypeptide lacking C-terminal (Table 1). The putatively deduced polypeptides were different (Fig. 8, Supplement File). Therefore, at least four troponin I isoforms existed in *S. chuatsi* white muscle and, we designated 171 aa putative full length polypeptide as TNNI1_SC, 154 aa putative peptide without N-terminal as TNNI2_SC, 131 aa putative peptide as TNNI3_SC and 175 aa putative peptide without C-terminal as TNNI4_SC.

When searching against Swiss-Prot database, four TNNI isoforms could be classified into two groups: TNNI1_SC, TNNI2_SC and TNNI3_SC belong to the first group and TNNI4_SC belong to second. The first group was significantly matched to Japanese quail *C. coturnix japonica* troponin I (TNNI2_COTJA: P68247) with identity from 50% to 57%, while TNNI4_SC was highly homologous to human *H. sapiens* troponin I with 72% identity (TNNI1_HUMAN: P19237) and rabbit *Oryctolagus cuniculus* troponin I (TNNI2_RABIT: P02643).

Because sequences between two groups were too flexible and within the first group too conserved, TNNI4_SC was aligned with other four vertebrates further. The further alignment identified the highly conserved minimal/main inhibitory peptide region, which reportedly interacted with actin and troponin C, and retained the important properties of intact troponin I such as inhibiting actomyosin ATPase activity (Syska et al. 1976; Zot and Potter 1987; Farah and Reinach 1995). The C-terminal over species was conserved too (Fig. 9, Supplement File). The C-terminal was also conserved among the sequences of the two groups. However, the corresponding minimal/main inhibitory peptide region in group 2 of troponin I was much dissimilar to that in group 1. The impacts of these differences on the inhibition of actomyosin ATPase activity were worthy of further exploration.

Troponin C

Three clusters were with complete ORFs encoding troponin C homologs in *S. chuatsi*. The most abundant cluster consisted of 84 ESTs, and the other two were composed of two ESTs each. The former cluster contained a complete ORF encoding a polypeptide of 160 aa long, and it was designated as TNNC1_SC. The second cluster of two ESTs also had a complete ORF encoding putatively yielded a polypeptide of 161 aa in length designated as TNNC2_SC. It was supposed that TNNC2_SC was the cardiac type because it was a minor isoform, which was one amino acid longer than skeletal troponin in mammals (Gillis and Tibbitts 2002). The third cluster of two ESTs, designated as TNNC3_SC, encoded the same full length polypeptide as TNNC1_SC.

As the major isoforms of troponin C in *S. chuatsi*, TNNC1_SC showed 79% identity with European eel *Anguilla anguilla* skeletal muscle troponin C, and relatively lower homology to troponin C in other vertebrates (72–74%), such as in edible frog *Rana esculenta* (74%), house mouse *M. musculus* (73%), rabbit *O. cuniculus*, and human *H. sapiens* (72%). However, the minor isoform TNNC2_SC was more similar, with identity ranging from 75% to 86%, to other vertebrates than TNNC1_SC (Seguchi et al. 2007).

Troponin C belongs to a Ca^{2+} -binding protein family that had four EF hands (Ca^{2+} -binding motif) labeled sequentially as I–IV. Each EF-hand comprised of a helix–loop–helix motif where Ca^{2+} could be potentially bound by oxygen ligands of six amino acids in the loop region at positions 1, 3, 5, 7, 9, and 12 (Dalla Libera et al. 1991). TNNC1_SC and TNNC2_SC were aligned with Ca^{2+} coordination sequences in other species. Multi-sequence analysis showed that EF-hands I and II were much more conserved than EF-hands III and IV. Ca^{2+} binding sites in EF-hands I and II motif over species were identical, while the replacement of F with Y took place in EF-hand III and K with M in EF-hand IV (Fig. 10, Supplement File). This was consistent with their functions. In skeletal troponin C, EF-hands I and II, of low affinity Ca^{2+} sites, regulated the interaction of troponin C with troponin I by binding Ca^{2+} to open a hydrophobic cavity where troponin C interacted with troponin I. However, C-terminal region, with EF-hands III and IV of high affinity Ca^{2+} sites, primarily served as binding domain for troponin I and provided free energy for binding troponin I (Grabarek et al. 1992).

Parvalbumin

Parvalbumins are soluble calcium-binding proteins that are especially abundant in fast-twitch muscles of fish. They aid muscle relaxation by competing with troponin C to bind the sarcoplasmic Ca^{2+} when Ca^{2+} is uptaken into the sarcoplasmic reticulum (SR) via SERCa (Berchtold et al. 2000). There were five clusters homologous to parvalbumin in the present study, each composed of 235, 157, 15, 1, and 1 ESTs. The former four clusters all had a complete ORF encoding 109 aa-long polypeptides with some variances in sequences, and they were designated as PRV1_SC, PRV2_SC, PRV3_SC, and PRV4_SC in sequence (GenBank accession number GU254023, GU254024, GU254025, and GU4026). The fifth cluster with a complete ORF encoding 108 aa-long polypeptides was designated as PRV5_SC. These five parvalbumin isoforms varied slightly from 4.46 to 4.65 in theoretical pI and from 11.47 to 11.96 kD in predicted molecule weight (Table 1). When searching against the Swiss-Prot database, the major isoform PRV1_SC showed the highest (75%)

homology to common carp *C. carpio* parvalbumin alpha (PRVA_CYPKA: P09227). The other isoforms PRV2_SC and PRV3_SC shared 85% and 80% identity with parvalbumin beta (P59747) of chub mackerel *Scomber japonicus*, respectively. The minor isoforms PRV4_SC also significantly matched to PRVA_CYPKA with 74% identity, while another isoform PRV3_SC showed 67% identity with zebra fish *Danio rerio* parvalbumin-7 (PRV7_DANRE: Q804W2).

Each parvalbumin molecule has two EF-hands, which showed high affinity for Ca^{2+} and moderate affinity for Mg^{2+} (Declercq et al. 1988). In alignment with other isoforms of parvalbumin, Ca^{2+} -binding regions were highly conserved. Ca^{2+} -binding region I was identical among all parvalbumin isoforms, but PRV3_SC and PRV5_SC were different between *S. chuatsi* and carp (Fig. 11, Supplement File). These differences would influence the flexibility of loops in EF-hands and the Ca^{2+} binding affinity in terms of Mg^{2+} binding affinity and Mg^{2+} dissociation rates, as demonstrated in other studies (Schneider et al. 2006). However, how these differences affected the Ca^{2+} binding affinity of parvalbumin was unknown and needed further biochemical studies.

Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase1

SERCa is an ATP-dependent Ca^{2+} pump that actively translocates Ca^{2+} into the SR prior to muscle relaxation (Jayantha Gunaratne and Vacquier 2007). Two clusters significantly matched to AT2A1_MAKNI in Atlantic blue marlin *Makaira nigricans* (AT2A1_MAKNI: P70083.2). The larger cluster of 30 ESTs was 3821 bp long termed as the major isoform SERCa1_SC. The region of 584–3025 bp of SERCa1_SC was significantly matched to that of 177–990 aa in AT2A1_MAKNI with 90% identity and the region of 159–584 bp to that of 1–142 aa in AT2A1_MAKNI with 81% identity. SERCa1_SC also had the 5' UTR, start codon, stop codon, and 3' UTR. The deduced polypeptide had 957 amino acid residues with a predicted mass of 105 kDa and showed the highest homology to AT2A1_MAKNI with 90% identity. However, the region of 143–176 in AT2A1_MAKNI did not exist in SERCa1_SC. The second cluster composed of nine ESTs significantly matched the N-terminal 343 amino acid residues in AT2A1_MAKNI with 82% identity, designated as SERCa2_SC. The lost region in SERCa1_SC was identified in SERCa2_SC (Fig. 12, Supplement File). The alignment of nucleotide sequences of SERCa1_SC and SERCa2_SC demonstrated that the deletion of 143–176 aa region may result from the alternative splicing (Fig. 13, Supplement file).

Multi-sequence alignment revealed that putative cytoplasmic domain (1–48 aa) was less conserved, and the deletion of 143–176 aa was located in another putative

cytoplasmic domain (111–253 aa). There was little information on the relationship between the sequences and the abilities of various SERCa isoforms to take back into sarcoplasmic reticulum. The impacts of the deletion of 143 to 176 aa fragment and other substitutions on the ability of SERCa to pump Ca^{2+} into the sarcoplasmic reticulum deserved further biochemical studies.

Conclusions

By analyzing the ESTs from the mandarin fish white muscle, we identified major isoforms of myofibrillar proteins and Ca^{2+} -handling proteins (SERCa and parvalbumin). We also found the homologs of muscle “glue” molecules and regulators of actin filament such as alpha-actinins, myosin-binding proteins, myomesin, tropomodulin, cofilin, profilin, twinfilins, coronin-1, and nebulin. The sequence data and its analysis would provide a basis for our systematic understanding of the biochemical, bioenergetic, and physiological diversity of the mandarin fish white muscle at molecular level and expand the utility of fish systems as models for the muscle function.

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