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Divergent evolution of the myosin heavy chain gene family in fish and tetrapods: evidence from comparative genomic analysis

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Ikeda D, Ono Y, Snell P, Edwards YJ, Elgar G, Watabe S. Divergent evolution of the myosin heavy chain gene family in fish and tetrapods: evidence from comparative genomic analysis. Physiol Genomics 32: 1–15, 2007. First published October 16, 2007; doi:10.1152/physiolgenomics.00278.2006.—Myosin heavy chain genes (MYHs) are the most important functional domains of myosins, which are highly conserved throughout evolution. The human genome contains 15 MYHs, whereas the corresponding number in teleosts appears to be much higher. Although teleosts comprise more than one-half of all vertebrate species, our knowledge of MYHs in teleosts is rather limited. A comprehensive analysis of the torafugu (Takifugu rubripes) genome database enabled us to detect at least 28 MYHs, almost twice as many as in humans. RT-PCR revealed that at least 16 torafugu MYH representatives (5 fast skeletal, 3 cardiac, 2 slow skeletal, 1 superfast, 2 smooth, and 3 nonmuscle types) are actually transcribed. Among these, MYHs, MYHs, and MYHs of fast and slow skeletal types, respectively, are expressed during development of torafugu embryos. Syntenic analysis reveals that torafugu fast skeletal MYHs are distributed across five genomic regions, three of which form clusters. Interestingly, while human fast skeletal MYHs form one cluster, its syntenic region in torafugu is duplicated, although each locus contains just a single MYH in torafugu. The results of the syntenic analysis were further confirmed by corresponding analysis of MYHs based on databases from Tetraodon, zebrafish, and medaka genomes. Phylogenetic analysis suggests that fast skeletal MYHs evolved independently in teleosts and tetrapods after fast skeletal MYHs had diverged from four ancestral MYHs.

Takifugu rubripes; syntenic analysis; gene cluster; whole gene duplication

MYOSINS ARE HIGHLY CONSERVED ubiquitous actin-based motor proteins that drive a wide range of motile processes in eukaryotic cells. So far, various types of myosins have been reported, and they are divided into 9–11 classes, where those classified into class II include the most extensively studied sarcomeric ones (reviewed in Ref. 3). The myosin molecule can be divided into two functional domains: an NH2-terminal globular head motor domain containing ATP- and actin-binding sites and a COOH-terminal α-helical coiled-coil rod domain having filament-forming ability. Two loop structures, loop 1 and loop 2, are located at the ATP- and actin-binding sites, respectively, in the globular head; thus both loops function essentially in the motor activity of myosin (3). In this regard, the myosin molecule consists of six subunits, two heavy chains (MYHs) of ~200 kDa and four light chains (MYLs) of ~20 kDa. Functional domains such as loop 1 and loop 2 are contained in MYH, whereas MYLs regulate the function of MYHs (3).

Analysis of the human genome reveals 11 sarcomeric MYH genes (MYHs), including 6 fast skeletal, 2 slow skeletal, 2 cardiac, and 1 superfast. It also contains 4 nonsarcomeric MYHs, including 3 nonmuscle and 1 smooth MYH, giving a total of at least 15 class II MYHs. In six fast skeletal MYHs, embryonic (MYH3) and perinatal (MYH8) MYHs are expressed during pre- and postnatal development of skeletal muscle, respectively (34), whereas three genes encoding types IIa (MYH2), IIb (MYH4), and IId/x (MYH1) are expressed primarily in adult fast skeletal muscle fibers. Extracardi MYH (MYH13) is expressed in extrinsic eye muscle only. Of the two genes encoding cardiac types-β (also known as type I, MYH7) and -α (MYH6), α-type MYH is expressed predominantly in heart, whereas β-type MYH is additionally expressed in slow skeletal muscle fibers.

Desjardins et al. (10) identified three novel sarcomeric MYHs in humans, two slow skeletal muscle isoforms, types A (MYH14/MYH7B) and B (MYH15), and one gene encoding superfasc MYH (MYH16), by molecular biological and database screening approaches. Three of the four nonsarcomeric MYHs, nonmuscle A (MYH9), nonmuscle B (MYH10) and smooth muscle types (MYH11), were identified more than a decade ago (9, 43, 46), whereas a novel one encoding nonmuscle C type (MYH14) recently has been described (17, 31). Because the nomenclature “MYH14” is also used for slow A type MYH (10), it seems that the name for nonmuscle C type should be changed to avoid confusion. While smooth muscle MYH restricts their transcripts in smooth muscle (2), those of nonmuscle MYHs are detected both in nonmuscle and muscle cells (45).

Although teleosts are the most successful group of vertebrates, composing more than one-half of all vertebrate species, our knowledge of MYHs in teleost is rather limited, and we do not even know the total number of MYHs in teleosts. Aside from mammalian MYHs, gene repertoire and genomic sequences of MYHs have been reported in common carp (Cyprinus carpio) (27, 37) and medaka (Oryzias latipes) (33). In sharp contrast to mammals with only 6 fast skeletal MYHs, 29 fast skeletal MYHs have been identified in common carp (27). A typical...
type of common carp MYH spans 11,385 bp, about one-half in size that of rat and chicken counterparts, because of the much smaller size of common carp introns (37). Interestingly, common carp express several types of fast skeletal MYHs encoding isoforms with different ATPase and motor activities following temperature acclimation (7, 19, 22, 55, 56). It has recently been found that grass carp (Ctenopharyngodon idellus) undergoes similar changes in temperature-dependent expressions of fast skeletal MYHs (49), although grass carp is a diploid fish compared with tetraploid common carp (39).

The genome database of tiger pufferfish, torafugu (Takifugu rubripes), was released publicly as the second example of a vertebrate after humans (1). Interestingly, retaining a gene repertoire similar to that of humans, the torafugu genome size is ~400 Mb, which is about eight times smaller than that of humans (4). The torafugu genome thus contains small sizes of introns and intergenic regions and is therefore ideal for comparative genomics (12, 54, 59). We have previously reported that torafugu contains one fast skeletal MYH in a syntenic region of the human MYH cluster and that other torafugu MYHs are somewhat scattered, unlike their mammalian counterparts, with two of them forming clusters (21, 57). McGuigan et al. (35) also traced the evolutionary lineage of fish sarcomeric MYHs including those of stickleback (Gasterosteus aculeatus), zebrafish (Danio rerio), and torafugu. However, the data were mainly obtained by in silico analysis, and little information on their transcripts is available. Since then, fish genome data have been improved for both zebrafish and the green spotted pufferfish (Tetraodon nigroviridis) (24). It has now become possible to analyze MYHs in more detail using these newly available public databases.

In the present study, we carried out a comprehensive analysis so as to obtain the complete catalog of fish sarcomeric and nonsarcomeric MYHs. For this purpose, we selected torafugu as the model organism, since it has the smallest vertebrate genome, which makes it ideal for searching the complete repertoire of MYH in teleost. Here, we report that torafugu contains at least 13 fast skeletal, 5 cardiac, 2 slow skeletal, 1 smooth, and 5 nonmuscle MYHs. Subsequent genomic structural analysis together with phylogenetic analysis suggests a divergent evolutionary lineage of fish and tetrapod MYHs.

MATERIALS AND METHODS

**T. rubripes.** Live specimen of adult torafugu (2.3 kg) grown at the Fisheries Laboratory of the University of Tokyo were used for collection of various tissues and extraction of total RNAs. Fertilized eggs prepared from wild torafugu (2–3 kg) were incubated in a 500-liter tank with running seawater at 18°C. Embryos were collected at various developmental stages for RNA extraction and for whole mount in situ hybridization.

In *silico* cloning of MYHs. The torafugu genome databases (version 3.0, mayffolds, and version 2.0, scaffolds, scaffold names of which start with “M” and “S,” respectively) (http://fugu.biology.qmul.ac.uk/blat/) were screened with the full-length amino acid sequence of a fast skeletal muscle MYH from carp (GenBank accession no. BAA22068) or human MYH10 (accession no. AAA99177) using tBLASTx. From various positive hits, we chose 41 regions in 36 scaffolds which were found to contain complete or partial MYHs. Their exon-intron structures were predicted with the WISE2 program (http://www.ebi.ac.uk/Wise2/) and confirmed by manual inspection. The recently released torafugu genome database (version 4.0), scaffold names of which start with “N,” was also used to verify the results obtained with the databases version 2.0 and version 3.0.

**Phylogenetic tree.** The deduced amino acid sequences of the head and rod domains of MYH, which are delineated by a conserved proline residue (838 of human MYH3; according to McGuigan et al., Ref. 35), were aligned using CLUSTAL W (51). Phylogenetic trees were constructed using the neighbor-joining (NJ) method on the software Mega3 (29). Bootstrap resampling analysis from 1,000 replicates was used to evaluate internal branches. The maximum-likelihood (ML) trees were reconstructed by the quartet puzzling algorithm using the TREE-PUZZLE (44). Reliability values (in percentage) for each internal branch expressed how often the corresponding cluster was found among the 1,000 intermediate trees.

**Expression analysis.** Total RNAs were extracted from fast muscle, slow muscle, heart, eye, intestine, and skin of an adult torafugu and hatching embryo (168 h postfertilization; hpf) using ISOGEN (Nippon Gene). First-strand cDNA was synthesized, and PCR was performed using primers specific to respective MYHs as described previously (20, 52). PCR was carried out successively with 5 cycles of 94°C for 30 s and 72°C for 1 min; 5 cycles of 94°C for 30 s, 72°C for 30 s, and 72°C for 1 min; and 25 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min, with a final extension step at 72°C for 10 min. All specific primer sequences and pairs to amplify MYH cDNAs encoding loop 2 region by RT-PCR are listed in Supplemental Tables S1 and S2, respectively (supplementary data are available at the online version of this article). In all RT-PCR, genomic DNA was used as a positive control. To confirm the amplification of targeted MYH cDNAs, all PCR products were subcloned into pGEM-T vector (Promega) and sequenced using a DNA sequencer (ABI PRISM 3100 Genetic Analyzer). The expression of MYH*<sub>390</sub>*s, which had no intron in the coding exon, was also confirmed using 3′-rapid amplification of cDNA ends (RACE) system (Invitrogen) with the gene-specific primer M880_3′RACE (Supplemental Table S1).

**Whole mount in situ hybridization.** Whole mount in situ hybridization (WISH) was performed according to Westerfield (58). RNA probes were synthesized from the pGEM-T vector incorporating cDNAs that encoded loop 2 regions of MYH*<sub>74a-2</sub>* and MYH*<sub>390</sub>*s. The full-length cDNAs encoding myogenin regulatory factors, myogenin (AB235115) and MyoD (AB235116), were subcloned into pGEM-T vector and used as probes.

**Construction of physical map.** The Ensembl genome browser (http://www.ensembl.org/) was used to determine the gene content and order of torafugu (Fugu 3.0, release 30.2e; Fugu 4.0, release 45.4f). Myofibrils (TETRAODON 7, release 45.4f), zebrafish (Zv4, release 30.4c; Zv6, release 45.6f), Tetraodon (TETRAODON 7, release 30.1b), medaka (HdiR, release 45.1b), and human (NCBI 36, release 45.36g). Torafugu bacterial artificial chromosome (BAC) and cosmide end sequences data were employed to connect the scaffolds that contained MYHs (http://fugu.biology.qmul.ac.uk/fugu-bin/clonesearch), the clone names of which start with “b” and “c,” respectively. Torafugu Scaffold Order and Orientation Display (http://fugu.biology.qmul.ac.uk/News/FuguWebsite_12_06_03.html) was also used to determine the physical relationships between scaffolds. The Display and end sequence databases are publicly available from the Queen Mary University of London via the web browser or the java application APOLLO (32).

**MYH content in BACs and cosmids.** The MYH content in cosmids and BAC clones was determined by classifying the variety of PCR products that encoded loop 1 and loop 2 regions. PCR was carried out using cosmid or BAC DNAs as templates and degenerated primer pairs, loop<sub>1</sub> F_all and loop<sub>1</sub> R_all for loop 1 and loop<sub>2</sub> F_all and loop<sub>2</sub> R_all for loop 2 (Supplemental Table S2), with 35 cycles of 94°C for 30 s, 45°C for 30 s, and 72°C for 1 min with a final extension step at 72°C for 10 min. The PCR products were subcloned into pGEM-T vector, and 48 clones were randomly selected and sequenced. The sequences obtained were aligned using CLUSTAL W, classified, and assessed for the number of MYHs in each cosmide and BAC clone.
**RESULTS**

**In silico cloning of MYHs.** We searched the torafugu genome database using amino acid sequences of common carp fast skeletal type and human nonmuscle type MYHs as probes and found 41 regions encoding the complete or partial MYHs in 36 scaffolds. Scaffolds M3256, M7684, M19402, and M26134 containing MYHs overlapped with clones found in scaffolds M343, S10198, M1034, and M1017, respectively, and, therefore, they were not considered for further analysis. Human sarcomeric MYHs have two 5′-untranslated exons, and the coding region in the last exon is only 3–8 bp. Because it was difficult to detect these untranslated regions using the in silico method, these exons were excluded from analysis. The results obtained are summarized in Fig. 1 and Supplemental Tables S3 and S4. To classify the types of MYHs, we used their intron positions, because genes sharing the same intron positions were thought to be homologous and closely related (Fig. 1). Finally, we found that torafugu has at least 28 MYHs, while mammals have a total of 15 (3, 10, 17, 31) (Table 1).

**Fast skeletal MYHs.** We extracted 18 regions containing fast skeletal type MYHs in 15 scaffolds, and two MYHs were found each in scaffolds M86, M743, and M2528 (Fig. 1). However, only 4 of 18 regions, M743–2, M2528–2, M454, and M1034, contained the complete sequence of MYHs. The sequences of 14 genes were incomplete either because of unread sequence part of the genome or their location flanking the scaffold sequence. To avoid overestimation of the gene number of torafugu fast skeletal MYH, we analyzed MYHs at the coding exon level and counted the maximum number of identical coding exons in every gene. As a result, we found that this fish contains at least nine fast skeletal MYHs as estimated from the presence of coding exons 30 and 31 (Fig. 1; M86–1, M86–2, M11037, M2528–1, M2528–2, M1034, M743–2, M454, and M10912).

The fast skeletal MYHs of torafugu are distributed in five genomic regions, with two regions containing a single MYH (scaffolds M454 and M1876) as also reported previously (21, 57). In the present study, three other regions were found to contain more than two MYHs, and we define them as cluster A for MYHs in scaffolds M939–M6536–M86, cluster B for those in M1034–M2528, and cluster C for those in M743 (Fig. 2).

These relationships of torafugu MYHs were confirmed by phylogenetic analysis in the present study (Fig. 3). Recently, the draft genome sequence of Tetraodon has become publicly available (24). Furthermore, information on the scaffold sequences of zebrafish has increased. In the present study, these genomic data were used to support the detailed physical map of torafugu MYHs as described below in detail.

**Fast skeletal MYHs in cluster A.** At least three fast skeletal MYH clusters are found in the telost genomes (Fig. 2, A–C) as also reported in our previous study (21, 57). Three scaffolds, M939, M6536, and M86, each containing partial sequences of MYH, were connected through analysis of BAC clones b193A04 and b218E09 (Supplemental Figs. S1A and S2A). To determine the number of MYHs in this cluster (cluster A), genomic sequences encoding loop 1 and loop 2 regions of MYH were amplified with degenerated primer sets using BAC clone b193A04. As a result, four different sequences were determined (loop 1: AB235135–AB235138; loop 2: AB235139–AB235142), suggesting that at least four MYHs are contained in cluster A (Fig. 2A). Although MYHs M11037 (Fig. 1) was incorporated into this cluster based on the sequence similarity, this gene was not considered as a single MYH because of the lack of adequate sequence information. Therefore, this cluster was regarded to contain at least four MYHs.

A syntenic region of torafugu cluster A in Tetraodon is dispersed into four scaffolds, S15050, S14470, S9104, and S9631. A long unread sequence gap of ~100 kb was found between KIAA1543 and PBX4 in S15050, indicating that three scaffolds, S14470, S9104, and S9631, with a total length of 70 kb are located in this unread sequence gap (Fig. 2A). However, it was difficult to determine the exact gene number in this syntenic region of Tetraodon, since MYHs are located in different scaffolds and only one MYH contains the complete coding sequence (data not shown).

Zebrafish also contains a syntenic region of torafugu cluster A, where complete and incomplete MYHs are tandemly arrayed (Fig. 2A).

**Fast skeletal MYHs in cluster B.** We previously reported that torafugu BAC end sequences of b230L18 connected two scaffolds, M2528 and M1034, in which two complete and one incomplete fast skeletal MYHs are tandemly arrayed in a head-to-head manner (21). In the present study, however, MYHs are arrayed in a head-to-head manner (Figs. 1 and 2B and Supplemental Fig. S2A). When genomic sequences encoding loop 1 and loop 2 regions of MYH were amplified from BAC clone b230L18, two MYH sequences each were found (loop 1: AB235143, AB235144; loop 2: AB235145, AB235146), suggesting that no MYH exists in the gap between M2528 and M1034 (Supplemental Fig. S2A). We then examined the possibility that scaffold M2528 is connected to another scaffold containing MYHs. Scaffolds M451, M6849, M3601, M2528, and M1034 were connected through BAC clones and scaffolds of version 2.0 assembly, confirming no MYHs in the scaffolds examined except M2528 and M1034 (Fig. 2B). Although MYHs M11033 (Fig. 1) was incorporated into this cluster based on the sequence similarity, this gene was not considered as a single MYH because of the lack of adequate sequence information. Thus cluster B was considered to contain at least three MYHs.

The Tetraodon syntenic region is found in S14979 with the number of MYHs and the direction of transcription being completely conserved between torafugu and Tetraodon (Fig. 2B). However, the exact number of MYHs in the Tetraodon syntenic region remains unclear because of unread sequence gaps between MYHs.

Zebrafish contains a syntenic region of torafugu M1034 in Zv6_S879, where only one MYH gene is found (Fig. 2B). The upstream region of MYH of zebrafish Zv6_S879 contains H1FX and ARPC4, which are not found in the syntenic region of torafugu and Tetraodon. The latter two species rather contain ACT1 and CACNA2D2 in the corresponding regions. A partial sequence of MYH containing coding exons 1, 3, and 4 on zebrafish Zv4_NA12812, which was not found in the Zv6 database, is highly similar, although not identical, to that of MYH on Zv6_S879 (data not shown). These results suggest that zebrafish has at least two MYHs that form fast skeletal MYH cluster B as in the case of pufferfish.
Fast skeletal MYHs in cluster C. The third fast skeletal MYH cluster, cluster C, is rather complex. Six cosmids, c109B14, c0602A21, c033B19, c064N04, c123C12, and c180B17, were mapped across cluster C (Supplemental Figs. S1 and S2). The cosmid end sequences of c109B14, and c0602A21y contained partial sequences of MYHM743-2, whereas c0602A21x, c033B19y, c064N04y, c123C12y, and c180B17y contained MYHM743-2 paralogs, although their transcriptional directions were opposite to that of MYHM743-2 (Supplemental Fig. S2B).

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When genomic sequences encoding loop 1 and loop 2 regions of MYH were amplified from c109B14 and c064N04, the former gave a single sequence each for loop 1 (AB235147) and loop 2 (AB235148). However, c064N04 provided two different sequences each for loop 1 (AB235149 and AB235150) and loop 2 (AB235151 and AB235152). It was noted that c0602A21 contained different partial sequences of MYH (AB235148). However, c064N04 provided two different sequences each for loop 1 (AB235147) and loop 2 (AB235148), respectively. The positions of the head and rod domains including the two loop regions are indicated at the top of Fig. 1. Only the coding exons (i.e., excluding the exons corresponding to the 5′- and 3′-noncoding regions) are used for comparison. Predicted torafugu clusters of fast skeletal and cardiac MYHs are indicated at left. Predicted human orthologous genes of slow skeletal, smooth, and nonmuscle MYHs are also indicated at left. Scaffolds marked by vertical lines at right of fast, slow, and smooth-nonmuscle type MYH notations are assumed to be the same gene. Exons with asterisks (*) correspond to the unread sequence part of the genome regions. Daggers (†) indicate the end of the scaffolds, whereas sharp (#) denote genomic regions where exons could not be predicted. Exons specific to torafugu where an adjacent intron is gained or lost when compared with the corresponding human orthologs are denoted by plus (+) and minus (−) symbols, respectively.
phylogenetic analysis. In addition, the two skeletal MYH sequences of Japanese lamprey (*Lethenteron japonicum*) reported recently (30) were aligned to consider the evolutionary relationship of MYHs, since it is well known that agnathan lampreys are derived from a common ancestor of gnathostome teleost and tetrapod. The topology obtained from the NJ tree is completely identical to that obtained from the ML tree (Fig. 4), separating lamprey MYHs as a distinct clade. MYH4454 and MYH1876 again formed one clade as described before (see Fig. 2). Taking into consideration the synteny of MYH4454 and MYH1876 in humans, it seems that these two MYHs have evolved from one common ancestor between teleosts and
tetrapods. We then defined these MYHs as fast D type, separating MYHs of fast A, B, and C types in clusters A, B, and C, respectively. Such an assumed evolutionary relationship will be further described in DISCUSSION.

Cardiac MYHs. Although eight regions in six scaffolds contained the sequences of cardiac MYHs, torafugu is thought to have at least five cardiac MYHs with only MYHM880 containing the complete coding exon (Fig. 1). As shown in Fig. 1, MYHM880 has no intron, and it seemed interesting to know whether this gene is functional (35). Thus we carried out 3'-RACE with a primer specific to MYHM880 and found that this gene is expressed only in cardiac muscle (Table 2), having at least two splice variants (AB235117 and AB235118). However, these splice variants do not alter the coding sequence, only the length of the untranslated region.

To examine the physical relationship between the scaffolds containing cardiac MYHs, we used the “Fugu Scaffold Order and Orientation Display” as well as BAC and cosmid end Fig. 2. Physical maps of human, torafugu, Tetraodon, zebrafish, and medaka syntenic regions containing fast skeletal MYHs. Gray and open rectangles indicate syntenic and nonsyntenic genes between fish and humans, respectively. MYHs of torafugu and humans are shown as black rectangles. MYHs of Tetraodon, zebrafish, and medaka are shown as black and checked rectangles with nos., which contain complete and incomplete genes, respectively. Genes displayed at right of midline are in forward strand (orientation, from top down), whereas those at left are in reverse strand (orientation, from bottom up). A–C: no MYH is present in the human syntenic regions. D: only a single MYH is contained in the torafugu and Tetraodon syntenic regions of human tissue containing fast skeletal MYH clusters. Although zebrafish syntenic regions of human MYH cluster were not found, we found corresponding regions in medaka (D). Arrowheads indicate the boundary between two scaffold sequences. Complete fast skeletal MYHs contain coding exons from 1 to 38, while the coding exon compositions in each MYH of Tetraodon, zebrafish, and medaka are as follows: no. 1 in A (A1), 33–38; A2, 29–38; A3, 1–38; A4, 19–27; A5, 25–28; A6, 25–32; A7, 10–17; A8, 1–25; A9, 1–38; A10, 32–33; B1, 14–38; B2, 6–38; B3, 1–10; B4, 1–38; B5, 1; 3–4; B6, 1–38; C1, 1–38; C2, 16–37; C3, 1–10; C4–C9; D1–D2, 1–38; D3, 9–14; 21–29; 34–38; D4, 21–38.

Fig. 3. The neighbor-joining tree based on deduced amino acid sequences in the head and rod domains of MYHs. The bootstrap values from a 1,000-replicate analysis are given at the nodes in percentage. Predicted clusters of torafugu fast skeletal and cardiac MYH isoforms and predicted human orthologs of slow skeletal, smooth, and nonmuscle MYH isoforms are shown at right. Refer to the legend of Fig. 4 for detailed description of fast A, B, C, and D. See INTRODUCTION for the description of slow A, slow B, nonmuscle A, nonmuscle B, and nonmuscle C. (See DISCUSSION for definitions of cardiac A and B.) Accession nos. for human MYHs used are as follows: MYH3, NM_002470; MYH2, AF117874; MYH1, AF117875; MYH4, AF117873; MYH8, NM_002474, MYH13, AF11782; MYH6, NM_002471; MYH7, NM_000257; MYH11, NM_002474; MYH9, NM_002473; MYH10, M69181; nonmuscle MYH14 (MYH17), AY165122. Amino acid sequences for MYH14, MYH15, and MYH16 have not been registered as single genes; thus their coding exons were predicted from the genomic sequences of NT_028392, NT_005612, and NT_007933, respectively.
sequence databases. The Display revealed that two BAC clones, b181K19 and b200J09, connected M187 to M2126, and scaffold S2358 connected M5502 to M1646 (Supplemental Fig. S1). In addition, b191B04 and c093P23 connected M1660 to M2126 and M1646, respectively, revealing that three scaffolds, M2126, M1646, and M5502, containing cardiac MYHs are tandemly arrayed and form a cardiac MYH cluster.

Another two cardiac MYHs, MYHM8248 and MYHM8560, are highly similar to the MYHs in M2126, and as a consequence these two genes were assimilated into this cluster. These results suggest that torafugu has at least two genomic regions containing cardiac MYHs, with one containing at least four MYHs.

Phylogenetic analysis shows that MYHM880 is probably an ancient cardiac MYH that existed before the divergence of teleosts and tetrapod (Fig. 3). However, no corresponding gene has been found from tetrapods so far. Cardiac MYHs of teleosts and tetrapods are located on the same clade (Fig. 3), implying that cardiac MYHs in each cluster have evolved from a common ancestor after their divergence.

We found that human orthologs of two genes close to MYHM2126, CHD8 and LOC161247, were also located near the human cardiac type MYH6 and MYH7 on 14q11.2 (Supplemental Fig. S3A), strongly suggesting that the MYHM2126 sequences are orthologs of human MYH6 and MYH7. In addition, although we found synteny between torafugu M880 (N268) and human 14q23.3, no MYH was found on human 14q23.3 (Supplemental Fig. S3A).

Slow skeletal and superfast MYHs. Four sarcomeric MYHs, MYHM3383, MYHS10198, and MYHM541, were defined as neither fast skeletal nor cardiac type. Intron positions and

Table 2. Expression of torafugu MYHs in various tissues and embryos

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<th>Accession No.</th>
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Fig. 4. The neighbor-joining (NJ; A) and maximum-likelihood (ML; B) trees based on deduced amino acid sequences encoded by rod domains of fast skeletal MYHs. The bootstrap values from a 1,000-replicate analysis are given at the nodes in percentage (A). Nos. indicate the quartet puzzling reliability values (B). Fast A, B, C, and D indicate the 4 fast skeletal MYH precursors, which might exist in the common ancestor of teleosts and tetrapods. See the legend to Fig. 3 for the accession nos. of human MYHs. MYHs of lamprey 1 and 2 and scallop were cited from BAD01606, BAD01607, and CAA39247, respectively.
phylogenetic tree strongly suggest that three of them, MYH5, MYHM383, and MYH10198, are of slow skeletal types and the remainder, MYHM541, of a superfast type (Figs. 1 and 3).

One of the three slow skeletal type MYHs, MYH5, contains all the complete coding exons, but the remaining two, MYHM383 and MYH10198, do not. However, it is likely that these two partial sequences are part of one gene, as these two sequences are highly similar to the Tetraodon orthologous gene (GSTENG00010022001, SCAF11841). The intron positions specific to MYHM383 and MYH10198, but not observed in MYH5 (see coding exons 22 and 27 in MYHM383 and coding exon 36 in MYH10198 in Fig. 1), are also highly similar to the Tetraodon orthologous gene. In fact, the torafugu genome database version 4.0 revealed that M383 and S10198 are connected by N398 and comprise a single gene (Fig. 1).

Detailed phylogenetic analysis suggests that the two slow skeletal type MYHs, MYHM383 and MYH10198, are the orthologous genes of human MYH14. Syntenic analysis strongly suggests that MYH14 is the human ortholog of torafugu MYHM5 (Supplemental Fig. S3B). Although there was no obvious synteny between MYHM383 and human MYH14 regions, human orthologs of the genes close to MYHM383 and S10198 were located near 21p11.2, the gene locus of MYH14. This result implies that, in addition to MYH5, MYHM383 and S10198 are also a torafugu paralog of human MYH14 resulting from the fish-specific genome duplication.

On the other hand, we could not find any ortholog of human MYH15 in any of the teleosts investigated, where the syntenic regions of MYH15 appeared to be highly scattered (Supplemental Table S5).

The orthologs of superfast MYH541 are found in zebrafish (ENSDARG000000034812, Zv4_scaffold14) and Tetraodon (GSTENG00013195001, SCAF14338), and the intron positions of the three genes are highly conserved. No synteny was found between human (17p13) and teleost MYH10 regions, several genes such as EVPL, SPHK1, and UBE2O mapped to 17q25.1 in humans are divided into two regions in teleost (Supplemental Fig. S3F). This implies whole genome or segmental duplication of ancestral MYH10 region and subsequent loss of redundant genes except MYH10 in teleost.

Only a single orthologous gene of human nonmuscle MYH17 (31) was found on scaffold M1017 of torafugu (Figs. 1 and 3). Again, while no obvious synteny was found between human MYH17 region (19p13.22) and torafugu M1017, some genes close to MYH17 are mapped to 19p13.22, implying that MYH17 and MYHM1017 are orthologs (Supplemental Fig. S3G).

Expression analysis. RT-PCR was carried out to examine the expression patterns of torafugu MYHs using primer sets specific to 16 MYHs (5 fast skeletal, 3 cardiac, 2 slow skeletal, 1 superfast, 2 smooth, and 3 nonmuscle types) (Supplemental Tables S1 and S2). To design a specific primer to each MYH, the loop 2 region, which has a hypervariable sequence and is thus thought to be easy to use to distinguish different genes, was chosen for all MYHs except the superfast type MYHM541 (18). In the case of MYH541, the genomic sequence information is available only for the rod region, and therefore a primer pair specific to this region was designed for this gene. Transcripts of all torafugu MYHs were detected in at least one tissue examined (Table 2, Supplemental Fig. S4).

Fast type MYHs tend to be expressed in adult fast and slow skeletal muscles, whereas cardiac types appear to be expressed only in adult slow skeletal muscle and heart. Slow type MYHs, on the other hand, are highly expressed in adult slow skeletal muscle and skin and also, in the case of MYH5, in adult fast skeletal and cardiac muscles. One of the two smooth muscle types, MYHM343, is expressed in all tissues examined, whereas MYHM541 is not found in heart, intestine, and embryos. Nonmuscle type MYHs are expressed in all tissues examined except MYH344, which is not found in adult fast skeletal muscle. Weak transcriptional signals of the superfast type are detected in heart, eye, and embryos. Interestingly, some fast type MYHs (MYH743-2, MYH252-2, and MYH454) are expressed in other tissues but not in adult fast skeletal muscle. For example, fast type MYH743-2 is strongly expressed in embryos, as in the case of slow type MYH5 (Table 2). Therefore, WISH was carried out using these two MYHs as probes to determine their detailed expression patterns in various embryonic stages.

Genes encoding myogenic regulatory factors such as MyoD and myogenin were used as positive controls for WISH. These two transcription factors are known to promote the expression of muscle-specific genes including MYHs (6). The transcripts of MyoD were observed at 72 and 96 hpf in slow skeletal muscle precursor cells called adaxial cells (Fig. 5, A and I) (50) and somites (Fig. 5, A, E, I, and M), whereas the myogenin transcripts were found only in somites (Fig. 5, B, F, J, and N). The expression signals of MyoD but not those of myogenin were detected in adaxial cells at 120 hpf (Fig. 5Q). The transcripts of fast type MYH743-2 and slow type MYH5 were detected following the expression of these myogenic factors, although their expression patterns are apparently different from each other. The transcripts of MYH743-2 were detected only in somites at all the stages tested (Fig. 5, C, G, K, O, and S).
However, those of slow type MYHs were found in adaxial cells and shifted to the surface of somites during development (Fig. 5, D, H, L, P, and T). In addition to skeletal muscle tissues, the transcripts of MYHs were detected in developing myocardium including bilateral heart precursor cells (Fig. 5, H and U), heart cone (Fig. 5V), and heart tube (Fig. 5, W and X), respectively.

**DISCUSSION**

**Repertoire of fish MYHs.** From scallop to mammals, a single MYH molecule of 200 kDa is composed of ~2,000 amino acids (3). However, the size of MYH is highly variable and depends on the intron size, resulting in human MYHs from ~30 kb for fast skeletal and cardiac types to 150 kb for nonmuscle MYH10 (3). The large size of the MYHs led to a lack of knowledge of repertoire and structure of this gene except in humans. However, information other than for humans is required to reveal the evolutionary lineage of vertebrate MYHs.

Recent progress in genome sequencing projects for torafugu, *Tetraodon*, zebrafish, and medaka have provided nearly complete genome sequences and structural models of MYHs. However, these models are based on single-genome sequences from a single species and cannot be used as a basis for the structural evolution of MYHs. Indeed, the sequence of MYHs from various species can be used as a basis for the structural evolution of MYHs.
plete catalogs of fish MYHs. The genomic sequence data disclose not only intron positions and phases but also the relative genomic positions and orientations of MYHs and their neighboring genes, which are necessary for syntenic analysis. While the molecular phylogenetic relationship based on the amino acid sequence between different species may be biased by their functions, syntenic analysis of orthologous genes seems to be independent of these effects, reflecting true evolutionary relationships. In the present study, three approaches including molecular phylogenetic, intron position, and syntenic analyses were employed to examine the relationship between teleost and tetrapod MYHs.

Whereas mammals have a total of 15 MYHs (3, 10, 17, 31), torafugu has at least 28, about twice as many as mammalian counterparts (Table 1). It is unlikely that new MYHs will be found in humans, where the genome has been comprehensively analyzed (23). In contrast to humans, it is highly probable that missing MYHs are still present in torafugu, where the genome database is still in progress. The presence of additional genes is at least in part because of teleost-specific genome duplication, including torafugu, Tetraodon, zebrafish, and medaka.

The results presented here reveal that torafugu has the complete repertoire of MYHs that have been found in humans with the exception of slow B type MYH15 (Table 1). It has been reported that MYH15 is the second ancient sarcomeric MYH, the divergence time of which is older than slow A type MYH14 (10, 35). As shown in Fig. 1, MYH14 lacks an intron between coding exons 34 and 35 compared with MYH3, -6, -7, -13, and -16. Phylogenetic analysis clearly shows that torafugu MYHMs and MYHs10198 are the duplicated orthologs of human MYH14 (see Fig. 3), but both have an intron at the same position between coding exons 34 and 35 as in MYH15 (see Fig. 1). It seems that the exon-intron structure of MYH14 was the same as that of MYH15 in the common ancestor of teleosts and tetrapods; subsequently, the descendant of MYH14 lost the intron after divergence in the human lineage. In fact, the Xenopus tropicalis orthologous gene of MYH14 (ENXSXETG00000007456, scaffold 38) also has an intron in the region preceding the coding exon 35. As shown in the expression analysis, MYHMs and MYHMs383 are functional (Table 2). If the ancient fish ortholog of MYH14 and MYH15 had similar functions, one of the duplicated MYH14s could take on the function of MYH15. If this is true, loss of a gene corresponding to MYH15 would have no disadvantage in the fish lineage.

Superfast type MYHs have been found from three fish species, torafugu, Tetraodon, and zebrafish. Although all of them have only partial sequence data, lacking the head domain in each database (Fig. 1), the gene appears functional in torafugu, at least at the transcriptional level (Table 2).

Thus we propose that the teleost/tetrapod ancestor probably possessed the complete set of MYHs, including fast and slow skeletal, cardiac, superfast, smooth, and nonmuscle types (see Table 1).

Additional four groups of MYHs of fish. Our results strongly suggest that the common ancestor of teleosts and tetrapods had at least four redundant groups of MYHs: three skeletal types (fast A, B, and C) and one cardiac type (cardiac A) MYH (Figs. 3 and 4 and Table 1). The descendants of the ancestral cardiac B type in teleosts and tetrapods form one clade (Fig. 3). Interestingly, the cardiac A type gene of torafugu, MYHM880, has no intron in its coding exon region, yet appears functional as revealed by its transcripts (Fig. 1, Table 2). This type of gene without any intron also has been reported in zebrafish (35) and might be found in other teleosts, the evolutionary and functional significances of which remain to be elucidated.

Expression patterns of torafugu MYHs. Figure 3 and Table 1 show that the 28 torafugu MYHs can be divided into 12 groups with no slow B type corresponding to MYH15. The expression analysis reveals that MYHs examined are expressed in at least one tissue described previously (Table 2).

MYHM743-2, one of the components in cluster C (Fig. 2), is highly expressed in hatching embryos but not in the adult (Table 2), and transcripts are found in somites (Fig. 5). The orthologous genes of MYHM743-2 have been also reported to be expressed at the embryonic stages in common carp (14, 38), zebrafish (5, 41, 60), and rainbow trout (42). Ennion et al. (14) reported that two fast skeletal MYHs named Eggs22 and Eggs24 are expressed during embryonic and larval development of common carp but not in juveniles or adults. The transcripts of Eggs22, but not those of Eggs24, were detected in the fast skeletal muscle fibers of developing lower jaw (14). Nihei et al. (38) have recently cloned two embryonic type MYHs, MYHemb1 and MYHemb2, and showed that their expression patterns are differentially regulated. Three different orthologous genes of torafugu MYHM743-2 have been reported in zebrafish (5, 41, 60). As was the case with common carp, the expression patterns of these zebrafish orthologs are not the same. These genes are also highly expressed at the embryonic or larval developmental stages in rainbow trout (42).

The transcripts of fast skeletal type MYHM1034, which falls under cluster B, are detected in both fast and slow skeletal muscles (Table 1). Similar fast skeletal type MYH has been cloned from the slow skeletal muscles of three-spine stickleback, Gasterosteus aculeatus (Gac R) (35), and yellowbelly rockcod, Nototenia coriceps (Nco R) (16). Bryson-Richardson et al. (5) also reported that zebrafish slow skeletal muscle expresses both slow and fast skeletal MYH isoforms. Thus it appears that the ancestral MYHs in cluster B of the evolutionary fast skeletal type seem to have diverged into a functional slow skeletal type through some evolutionary process, although phylogenetic analysis clearly separates the fast and slow type MYHs into two distinct clades.

MYHMs66-1, a component in fast cluster A, is also expressed in fast and slow muscles (Table 2). Fernandes et al. (15) have recently constructed cDNA libraries from torafugu fast skeletal muscle, yielding 1,452 expressed sequence tags (ESTs). We found that 154 of 1,452 ESTs represented transcripts of MYHs, where 135 ESTs were transcribed from MYHs in fast cluster A and 19 from those in cluster B (15). These results suggest that MYH isoforms in fast cluster A are the main components in adult torafugu fast skeletal muscle. On the other hand, MYHs mainly expressed in adult fast skeletal muscle of the greater amberjack, Seriola dumerili, and hawkfish, Paracirrhites forsteri (35), are the orthologs of those in fast cluster A, whereas MYHs expressed in N. coriceps and white croaker, Pennahia argentata, form a clade with that in fast cluster C.

MYHM454 is not the main component in fast skeletal muscle but is rather abundant in eye and intestine (Table 2). The physical map and phylogenetic analysis clearly show that MYHM454 and MYHM1876 are the duplicated orthologous genes of tetrapod fast skeletal MYHs (Figs. 2D and 5). It was noted...
that neither of the two genes formed any cluster with other fast skeletal MYHs (Fig. 2D). MYHs in clusters A, B, and C might play a major role in fast skeletal muscle in teleost, and there would be very low selective pressure on MYHM454 and MYHM1876 to retain the function in fast skeletal muscle, although the two genes might have extended their function in the other tissues such as eye and intestine, as evidenced from the presence of those transcripts in these tissues. In fact, the evolutionary rates of these two genes are faster than for other MYHs (Figs. 3 and 4), and no orthologs to MYHs in teleost and tetrapod. The MYHs in these clusters have no synteny with the human fast MYHs (data not shown), which might be defined as a cardiac/slow type. The present study shows that not only fast but also slow skeletal muscle fibers might express different types of MYHs, cardiac/slow type and slow type MYHs. It seems important to distinguish the functions of different fibers belonging to the same muscle type but expressing different MYHs.

It has been reported that bilateral heart precursor cells located in the region of hindbrain migrate medially and merge to construct a horseshoe-shaped structure (heart cone) in zebrafish (47, 48). The cone forms a heart tube, and this simple tube then undergoes complex morphogenetic movements that lead to the formation of heart (47). Interestingly, the transcripts of slow type MYHM5 are found in developing heart and detected in bilateral heart precursor cells, heart cone and heart tube (Fig. 5, H, U–X).

So far, nonsarcomeric MYHs have not been studied in teleosts. The two duplicated smooth muscle type MYHs of torafugu exhibit different tissue distribution in their expression. MYHM454 is expressed in all tissues examined, whereas the transcripts of MYHM581 are predominantly found in eye (Table 2). The transcripts of mouse smooth muscle MYH are completely restricted to smooth muscles that exist in blood vessels and digestive organs throughout development (36). The transcripts of three nonmuscle type MYHs of torafugu are found in almost all torafugu tissues examined including eye, intestine, and skin (Table 2).

Plausible evolutionary histories of fast skeletal MYHs. In the present study, MYHs in fish and mammals are suggested to be formed independently after divergence from a common ancestor. In fact, the syntenic analysis strongly suggests that torafugu MYHM454 and MYHM1876 and six human fast skeletal MYHs were diverged from a common ancestor (Fig. 2D). If all torafugu fast skeletal MYHs distributed to the five loci were diverged from a common ancestor after the divergence of teleosts and tetrapods, these loci all would exhibit synteny with one cluster of human fast skeletal MYH. However, three torafugu MYH clusters have no synteny with the human fast
skeletal MYH cluster (Fig. 2, A–C). It has been reported that the divergence between ray-finned fish (teleost lineage) and lobe-finned fish (tetrapod lineage) occurred 450 million years ago (28) followed by a whole genome duplication in teleost that occurred 320–350 million years ago (8, 53). Our analyses suggest the most plausible scenario, that at least four types of fast skeletal MYHs were present in the common ancestor of teleosts and tetrápods (Fig. 6). Subsequently, three fast skeletal MYHs (fast A, B, and C groups) were lost in the tetrapod lineage, and the remaining fast D group of MYH gave rise to the fast skeletal MYH cluster in humans (Fig. 6). The quadruplicated orthologs of ancient tetrapods with similar functions might have permitted three of the four to be deleted in their evolutionary lineage. In teleosts, all four ancestral fast skeletal MYHs have been retained and have undergone a whole genome duplication giving rise to eight MYHs (Fig. 6). Subsequently, three of the eight duplicated MYHs have been lost during the course of evolution. Later on, lineage-specific tandem duplication occurred in three of the remaining five duplicated MYHs, resulting in three MYH clusters (Fig. 6).

X. tropicalis contains a single syntenic region of human MYHs of the fast D group but not of the fish fast A, B, and C groups, as revealed in scaffold 255 of the Ensembl database (version 39.41a, http://www.ensembl.org/). This fact implies that the formation of fast skeletal MYH cluster and concomitant loss of MYHs corresponding to those of fish fast A, B, and C groups in tetrápods occurred before the divergence of amniotes and amphiobians. It will be of interest to analyze MYHs of other ray-finned fishes and other teleosts in lower phylogenetic positions.

Our evolutionary scenario of fast skeletal MYHs in teleosts is consistent with the model of teleost genome evolution described by Kasahara et al. (26). In their model, the number of protochromosomes before the whole genome duplication event is estimated to be 13 (26). We found that medaka fast D group MYHs were located at 8: 2.54M and 19: 19.61M (Fig. 2D), and both of these loci were thought to be derived from protochromosome “e” (Supplemental Table S6) (26). In addition, human fast skeletal MYH locus 17p13.1 is also thought to be derived from protochromosome e (Supplemental Table S6) (26). Our analysis also suggests that, in medaka, MYHs of fast A (8: 8.93M), B (5: 3.13M), and C (6: 20.15M) are derived from protochromosomes e, “l,” and “j” or “k,” respectively (Supplemental Table S6) (26). These results suggest that four types of MYHs were divided onto three protochromosomes, A and D on e, B on l, and C on j or k, before the whole genome duplication (Supplemental Table S6).

It is difficult to speculate why MYHs of fast D and fast A, B, and C types were selected to build cluster(s) in tetrápod and teleost, respectively. These clusters are found to be formed repeatedly during vertebrate evolution, which implies that the increase of the MYH repertoire might be necessary for the complex musculature system accompanying the morphological evolution, and that selective expression of individual or a group of MYHs could be tightly regulated if they are clustered in the genome. However, in teleost lineage, not only the number of MYHs but also the number of MYH loci (clusters) had increased. One of the most plausible reasons is that, as poikilothermic organisms, teleosts had to survive and evolve in aquatic habitats often characterized by large variations in environmental parameters including temperature as the most critical one. Evolutionary pressure to maintain a normal physiology including locomotion and speed at various temperatures thus allowed teleosts to acquire a larger MYH repertoire than homoeothermic tetrápods. In fact, we found that common carp (7, 22, 19, 55, 56), grass carp (49), and medaka (33) express several types of fast skeletal MYHs in response to temperature change. In this regard, it is worth noting that eurythermam temperate fish such as common carp (27) and medaka (33) have more MYHs than torafugu, at least in cluster C, further substantiating the fact that the number of MYHs is related to a particular lineage or species-specific physiological needs. Comprehensive data on the MYH repertoire in poikilothermic tetrápods such as amphibians and reptiles are necessary to obtain further insight in this regard.

In conclusion, we comprehensively analyzed torafugu sarcoemeric and nonsarcomeric MYHs and studied their evolutionary histories compared with mammalian MYHs as well as Tetraodon, zebrafish, and medaka MYHs. Torafugu contains at least 13 fast skeletal, 5 cardiac, 2 slow skeletal, 1 superfast, 2 smooth, and 5 nonmuscle MYHs; this torafugu MYH repertoire is in marked contrast to that of human tissue, which contains 6 fast skeletal, 2 cardiac, 2 slow skeletal, 1 superfast, 1 smooth, and 3 nonsarcomeric MYHs. RT-PCR reveals that 16 MYHs representing 5 fast, 3 cardiac, 2 slow, 1 superfast, 2 smooth, and 3 nonmuscle types are actually transcribed. The present study suggests that fast skeletal MYHs evolved independently in teleosts and tetrápods after fast skeletal MYHs had diverged from a common ancestor of sarcomeric MYHs.

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