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Temperature and the expression of myogenic regulatory factors (MRFs) and myosin heavy chain isoforms during embryogenesis in the common carp *Cyprinus carpio* L.

Nicholas J. Cole[1,†], Thomas E. Hall[2,*,†], Christopher I. Martin[2], Mark A. Chapman[3], Atsushi Kobiyama[4], Yoshiaki Nihei[5], Shugo Watabe[5] and Ian A. Johnston[2]

[1]*Division of Cell and Developmental Biology, MSI/WTB Complex, University of Dundee, Dow Street, Dundee, DD1 5EH, UK,* [2]*Gatty Marine Laboratory, School of Biology, University of St Andrews, St Andrews, Fife KY16 8LB, UK,* [3]*Sir Harold Mitchell Building, School of Biology, University of St. Andrews, Fife, KY16 9TH, UK,* [4]*Laboratory of Aquatic Microbiology, School of Fisheries Sciences, Kitasato University, Sanriku, Iwate 022-0101, Japan* and [5]*Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo, Tokyo 113-8657, Japan*

*Author for correspondence at present address: Victor Chang Cardiac Research Institute, 384 Victoria Street, Darlinghurst, Sydney, NSW 2010, Australia (e-mail: t.hall@victorchang.unsw.edu.au)

†These authors contributed equally to this work

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Summary

Embryos of the common carp, *Cyprinus carpio* L., were reared from fertilization of the eggs to inflation of the swim bladder in the larval stage at 18 and 25°C. cRNA probes were used to detect transcripts of the myogenic regulatory factors MyoD, Myf-5 and myogenin, and five myosin heavy chain (MyHC) isoforms during development. The genes encoding Myf-5 and MyoD were switched on first in the unsegmented mesoderm, followed by myogenin as the somites developed. *Myf*-5 and *MyoD* transcripts were initially limited to the adaxial cells, but *Myf*-5 expression spread laterally into the presomitic mesoderm before somite formation. Two distinct bands of staining could be seen corresponding to the cellular fields of the forming somites, but as each furrow delineated, *Myf-5* mRNA levels declined. Upon somite formation, *MyoD* expression spread laterally to encompass the full somite width. Expression of the *myogenin* gene was also switched on during somite formation, and expression of both transcripts persisted until the somites became chevron-shaped. Expression of *MyoD* was then downregulated shortly before *myogenin*. The expression patterns of the carp myogenic regulatory factor (MRF) genes most-closely resembled that seen in the zebrafish rather than the rainbow trout (where expression of *MyoD* remains restricted to the adaxial domain of the somite for a prolonged period) or the herring (where expression of *MyoD* persists longer than that of *myogenin*). Expression of two embryonic forms of *MyHC* began simultaneously at the 25–30 somite stage and continued until approximately two weeks post-hatch. However, the three adult isoforms of fast muscle MyHC were not detected in any stage examined, emphasizing a developmental gap that must be filled by other, as yet uncharacterised, MyHC isoform(s). No differences in the timing of expression of any mRNA transcripts were seen between temperature groups. A phylogenetic analysis of the MRFs was conducted using all available full-length amino acid sequences. A neighbour-joining tree indicated that all four members evolved from a common ancestral gene, which first duplicated into two lineages, each of which underwent a further duplication to produce *Myf-5* and *MyoD*, and *myogenin* and *MRF4*. Parologous copies of *MyoD* from trout and *Xenopus* clustered closely together within clades, indicating recent duplications. By contrast, *MyoD* paralogues from gilthead seabream were more divergent, indicating a more-ancient duplication.

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Key words: *Cyprinus carpio*, temperature, development, muscle, *in situ* hybridization, carp, phylogeny, myogenic regulatory factor, MRF.

Introduction

The myogenic regulatory factors (MRFs) are a family of basic helix–loop–helix (bHLH) transcription factors essential to the specification and determination of the muscle cell lineage. The four members of this protein family, MyoD, Myf-5, myogenin and MRF4, are characterised by their ability to induce myogenic conversion in a variety of

cell types, including fibroblasts, neurons, adipocytes, chondrocytes and melanocytes (reviewed by Edmondson and Olsen, 1993; Arnold and Braun, 2000). The bHLH domain is central to the role of transcriptional activation and is highly conserved, with the four proteins sharing approximately 80% amino acid sequence identity in this region within species (Edmondson and Olsen, 1993). The HLH region is characterised by two amphiphatic α-helices, separated by an unstructured intervening loop. HLH regions are mutually attractive and facilitate the formation of functionally active protein dimers (Maleki et al., 2002). The basic region forms an extension of one of the α-helices of the HLH region and facilitates DNA binding. bHLH dimers specifically bind E-box elements (CANNTG) found in the promoters and enhancers of most, if not all, muscle-specific genes (Apone and Hauschka, 1995; Spinner et al., 2002) although it is likely that nucleotide variation in the flanking regions and within the motif imparts some specificity (Ludolph and Konieczny, 1995).

During myogenesis, the transcription factors Myf-5 and MyoD are required for the initial determination of the myogenic lineage. Gene knockout studies in mice show that lack of MyoD and Myf-5 results in failure of myoblast formation, and a consequent lack of all head and trunk skeletal muscle (Rudnicki et al., 1993). In zebrafish, targeted knockdown with a *Myf-5* morpholino has been shown to induce defects in myogenesis and brain formation (Chen and Tsai, 2002). The expression of *myogenin* and *MRF4* is activated during myoblast differentiation (Rhodes and Konieczny, 1989; Wright et al., 1989; Miner and Wold, 1990; Edmondson and Olson, 1993; Pownall et al., 2002), and myogenin and MRF4 probably have cooperative functions with MyoD and Myf-5 as transcription factor regulators for the activation of muscle contractile protein genes (Lassar et al., 1991). In myogenin-knockout mice, myoblasts form in the correct place but do not fuse into muscle fibres (Hasty et al., 1993; Nabeshima et al., 1993; Venuti et al., 1995). The function of MRF4 is less clear because in all three mutants constructed to inactivate it, Myf-5 production is also affected (Olson et al., 1996; Summerbell et al., 2000, 2002).

In the zebrafish, *Danio rerio*, *Myf-5* and *MyoD* transcripts are initially seen at approximately 7.5 h at 28.5°C (80% epiboly) in bilateral bands of cells flanking the presumptive notochord (Weinberg et al., 1996; Chen et al., 2001; Coutelle et al., 2001). The expression patterns of these two genes overlap considerably, incorporating the adaxial cells as they form. Expression of *Myf-5* extends further into the presomitic mesoderm than that of *MyoD* but, strikingly, as the adaxial cells become incorporated into the somites, *Myf-5* transcription dramatically declines. Expression of *MyoD* persists in the differentiated somites until much later, after they become chevron-shaped, whereupon it is downregulated. Expression of *myogenin* begins at 10.5 h (at 28.5°C) in a subset of the *MyoD*/*Myf-5*-expressing cells (Weinberg et al., 1996; Chen et al., 2000). The *myogenin* transcripts first appear in bands of cells extending laterally away from the adaxial cells. However, this lateral extension of expression is narrower than in the case of *MyoD* and, due to its later onset, first expression is within the somites rather than the presomitic mesoderm. Transcription of *myogenin* is also transient, and persists until shortly after the disappearance of *MyoD* transcription. Furthermore, there are some differences in MRF gene expression between fish species. In the rainbow trout, *Oncorhynchus mykiss*, for instance, *MyoD* expression, rather than spreading laterally, remains confined to the medial domain of the somite for a prolonged period (Delalande and Rescan, 1999). In the herring *Clupea harengus*, *myogenin* mRNA shows a more transient expression pattern than that seen in zebrafish (Weinberg et al., 1996) and trout (Delalande and Rescan, 1999), disappearing from the somites before the downregulation of *MyoD* (Temple et al., 2001). A number of species including the trout (Rescan and Gauvry, 1996), gilthead seabream *Sparus aurata* (Tan and Du, 2002) and *Xenopus laevis* (Scales et al., 1990; 1991; Charbonnier et al., 2002) also possess multiple copies of one or more MRF-encoding genes.

Temperature has been shown to influence many aspects of development in teleosts, including muscle cellularity (Stickland et al., 1988; Vieira and Johnston, 1992; Nathanailides et al., 1995; Johnston and McLay, 1997; Matschak et al., 1998; Galloway et al., 1998, 1999; Hall and Johnston, 2003) and the relative timing of myofibrillogenesis (Johnston et al., 1995, 1996, 1997). There is also a small body of evidence to suggest the timing and extent of MRF gene expression varies with temperature. Xie et al. (2001) detected *MyoD* and *myogenin* mRNAs in a greater number of somites in trout embryos of the same developmental stage, reared at 12°C compared with 4°C. This change in expression was apparently concomitant with a 'relatively advanced' state of muscle development at 12°C compared with 4°C. Similarly, Wilkes et al. (2001) used quantitative northern blots to show that *MyoD* and *myogenin* mRNA levels in trout and sea bass *Dicentrarchus labrax* were highest at temperatures close to those of the usual environmental spawning temperatures for the species. By contrast, Temple et al. (2001) found no difference in the timing of *MyoD* or *myogenin* expression in herring embryos reared at 5, 8 and 12°C. Hall et al. (2003) also found no difference in the timing of *MyoD* expression between Atlantic cod *Gadus morhua* embryos reared at 4, 7 and 10°C, although the timing of blastopore closure relative to somite stage was relatively delayed at 7 and 10°C when compared with 4°C, and the number of deep fibres at hatching in the 10°C group was significantly higher than in the lower temperature groups.

Fishes from cold environments express myosin heavy chain (MyHC) protein isoforms with a higher specific myofibrillar ATPase activity and a lower thermal stability than those from warmer environments (Johnston et al., 1973, 1975a,b), and there is an apparent trade off between these traits. Species with a broad temperature tolerance, such as the goldfish *Carassius auratus* and the common carp *Cyprinus carpio*, can alter their Mg^{2+} Ca^{2+} ATPase activity depending on the ambient

temperature by differential expression of multiple *MyHC* genes (Goldspink et al., 1992; Watabe et al., 1995; Imai et al., 1997; Cole and Johnston, 2001). The control of such acclimation responses is unknown and, to date, has not been demonstrated in embryos, which express many of their own developmental stage-specific isoforms of muscle proteins (Scapolo et al., 1988; Crockford and Johnston, 1993; Johnston et al., 1997). In mammals, there is evidence for involvement of the MRFs in the determination of contractile protein isoform expression and fibre typing (Voytik et al., 1993; Hughes et al., 1999) along with other influences, such as hormones and innervation (Hughes et al., 1993; Lefeuvre et al., 1996).

The common carp is a eurythermal species commonly inhabiting waters that fluctuate between near freezing and 30°C seasonally (Michaels, 1988). Spawning occurs in the summer months at a minimum temperature of ~18°C, and the eggs and larvae develop normally between temperatures of 18 and 25°C (Penáz et al., 1983; Balon, 1995). In the presnt study, the spatial and temporal expression patterns of *MyoD*, *myogenin*, and *Myf-5* were characterised, and the hypothesis that temperature influences expression of the MRFs within the normal limits of thermal tolerance was investigated by comparing embryos and larvae reared at 18 and 25°C. The *in situ* expression pattern of *Myf-5* was of particular interest because within the Teleostei, to date, it has only been described in the zebrafish and has never been investigated in relation to temperature. In addition, the expression of five different *MyHC* transcripts (two embryonic types, Ennion et al., 1999; and three temperature-specific types, Imai et al., 1997) were characterised and compared between temperature groups. The aims of the present study were to investigate the initial expression of temperature-specific *MyHC* isoforms in larvae, and whether embryonic isoforms are differentially expressed in response to rearing temperature, and to characterise the timing of expression switching from embryonic to adult isoforms. Finally, since many MRF cDNAs from teleosts have been cloned in recent years and parologous genes have been identified, a comprehensive phylogeny of vertebrate MRFs was also undertaken. Neighbour-joining and parsimony analyses were used to generate phylogenies to elucidate evolutionary relationships between the genes and the relative timing of gen(om)e duplication events.

Materials and methods

Spawning and larval rearing

Carp spawning and egg incubation were carried out according to Michaels (1988). Briefly, over-wintering adult carp were brought into the laboratory in early January 2000. The water temperature was raised by 3°C per day from 4 to 25°C, where it was held for a further six weeks. Female fish were given 0.6 mg kg^{-1} of carp pituitary acetone powder (Sigma, Poole, UK) by intramuscular injection, followed by 3 mg kg^{-1} 12 h later. Males were given a single injection of 1.5 mg kg^{-1}. After a further 12 h, eggs and milt were stripped into separate dry containers. They were mixed in the ratio 1:100 (v/v) milt:eggs, and activated with an equal volume of 0.3% urea, 0.3% NaCl. The fertilization reaction was allowed to proceed for 1 h, after which the eggs were washed three times in 0.5% (v/v) tannic acid to prevent aggregation. Fertilized eggs were transferred to Zuger jars and incubated under constant aeration at 18 and 25°C±1°C (range). Embryos were sampled every 6 h by anaesthetizing in 0.1% (m/v) tricaine (MS-222; Sigma, Poole, UK), puncturing the chorion with a hypodermic needle, and fixing in 4% (m/v) paraformaldehyde in phosphate-buffered saline (PBS). After 12 h of fixation the embryos were washed once in PBS and stored at –80°C in 100% methanol.

Plasmid clones and cRNA probes

The *MyoD*, *myogenin* and *Myf-5* clones used were as previously described by Kobiyama et al. (1998). 10°C-type, intermediate-type, and 30°C-type *MyHC* were as described by Imai et al. (1997). The two embryonic-type *MyHC* clones (*Eggs22* and *Eggs24*) were generously supplied by Geoff Goldspink and are described by Ennion et al. (1999). DIG-labelled cRNA probes were constructed from linear plasmids according to Hall et al. (2003). Details of plasmids, restriction endonucleases and transcriptases are shown in Table 1.

In situ *hybridization*

Five embryos of equivalent developmental stages from each sample were selected per cRNA probe. *In situ* hybridization was carried out using the procedure described by Hall et al. (2003). Photographs were taken on a Leica MZ7.5 binocular microscope (Leica, Milton Keynes, UK) using darkfield illumination and a Zeiss Axiocam imaging system (Zeiss, Welwyn Garden City, UK).

Table 1. *Details of* MRF *and* MyHC *clones, and modifying enzymes used*

Gene name	GenBank Accession no.	Clone length (nt)	Plasmid	Sense endonuclease	Antisense endonuclease	Sense transcriptase	Antisense transcriptase
MyoD	AB012882	1221	pBluescript SK^-	*Xho*I	*Spe*I	T3	T7
Myogenin	AB012881	855	pBluescript SK^-	*Not*I	*Xho*I	T7	T3
Myf-5	AB012883	500	pBluescript SK^-	*Not*I	*Xho*I	T7	T3
MyHC Eggs22	AJ009735	161	pBluescript$^+$	*Eco*RI	*Hin*dIII	T3	T7
MyHC Eggs24	AJ009734	152	pBluescript$^+$	*Eco*RI	*Hin*dIII	T3	T7
MyHC 10°C-type	D50474	232	pBluescript SK^-	*Not*I	*Xho*I	T7	T3
MyHC Intermediate-type	D50475	523	pBluescript SK^-	*Not*I	*Xho*I	T7	T3
MyHC 30°C-type	D50476	254	pBluescript SK^-	*Not*I	*Xho*I	T7	T3

RNA dot-blotting

Total RNA was extracted from the trunk muscle of hatched larvae (the head, tail and yolk sac were removed) using Tri-reagent (Sigma, Poole, UK). RNA dot-blots were performed by spotting 2.5 µg of total RNA in 0.5 µl water onto nitrocellulose (Hybond-N$^+$; Amersham-Pharmacia, Little Chalfont, UK), and fixing at 120°C in an oven for 30 min. A 30 min prehybridization was carried out in 50% (v/v) formamide, 0.1% (m/v) N-lauroylsarcosine, 0.02% (m/v) SDS, 2% (v/v) blocking reagent (Roche, Lewes, UK) at 65°C, before addition of probe at 100 ng ml^{-1}. After hybridization overnight at 65°C, the blots were washed 2×15 min in 2× SSC, 0.1% (m/v) SDS at room temperature, followed by 2×15 min in 0.5× SSC, 0.1% SDS at 65°C. Membranes were blocked in 2% (v/v) blocking reagent, 100 mM maleic acid, 150 mM NaCl, pH 7.5 for 1 h, before addition of an alkaline-phosphatase-conjugated anti-DIG antibody, Fab fragments (Roche, Lewes, UK) at a dilution of 1/100,000. After a 30 min incubation in the antibody solution,

Fig. 1. Expression of myogenic regulatory factors and embryonic myosin heavy chain isoforms in common carp embryos reared at 18 and 25°C during development. Scale bars, 1 mm (a) *Myf-5* 18°C, (b) *Myf-5* 25°C, (c) *MyoD* 18°C (d) *MyoD* 25°C, (e) *myogenin* 18°C, (f) *Myogenin* 25°C, (g) *MyHC Eggs22* 18°C, (h) *MyHC Eggs22* 25°C, (i) *MyHC Eggs24* 18°C, and (j) *MyHC Eggs24* 25°C. (i) Completion of epiboly, before somite formation, (ii) ~15-somite stage, (iii) ~23-somite stage, (iv) ~30-somite stage, (v) completion of somitogenesis (38 or 39 somites), (vi) hatched larvae.

membranes were washed 2×15 min in 100 mM maleic acid, 150 mM NaCl, pH 7.5, 0.3% (v/v) Tween-20. Detection was achieved using a 1:100 dilution of the chemiluminescent substrate CSPD (Roche, Lewes, UK), in 100 mM Tris-HCl, 100 mM NaCl, pH 9.5 followed by exposure to X-ray film.

Phylogenetic analysis of MRF sequences

A phylogenetic analysis was undertaken using full-length amino acid sequences of vertebrate MRFs taken from the GenBank database (NCBI, Bethesda, USA). Five additional sequences were predicted from Ensembl (www.ensembl.org) and Genoscope (www.genoscope.cns.fr) genome assemblies (see Data 1 in supplementary material), using Blast2 (v2.2.6) (Altschul et al., 1997) and Genewise (Birney et al., 2004). An initial multiple alignment was constructed using the Clustal algorithm in Lasergene (DNAstar Inc., Madison, USA), which was then improved by eye. A neighbour-joining (NJ) tree was constructed in PHYLIP (Felsenstein, 1995) and bootstrapped 1000 times to provide statistical support. Parsimony analysis was carried out using PAUP (Swofford, 2002) (see Data 2 in supplementary material).

Results

Somitogenesis began almost immediately following epiboly as described by Verma et al. (1970) and Penáz et al. (1983), at

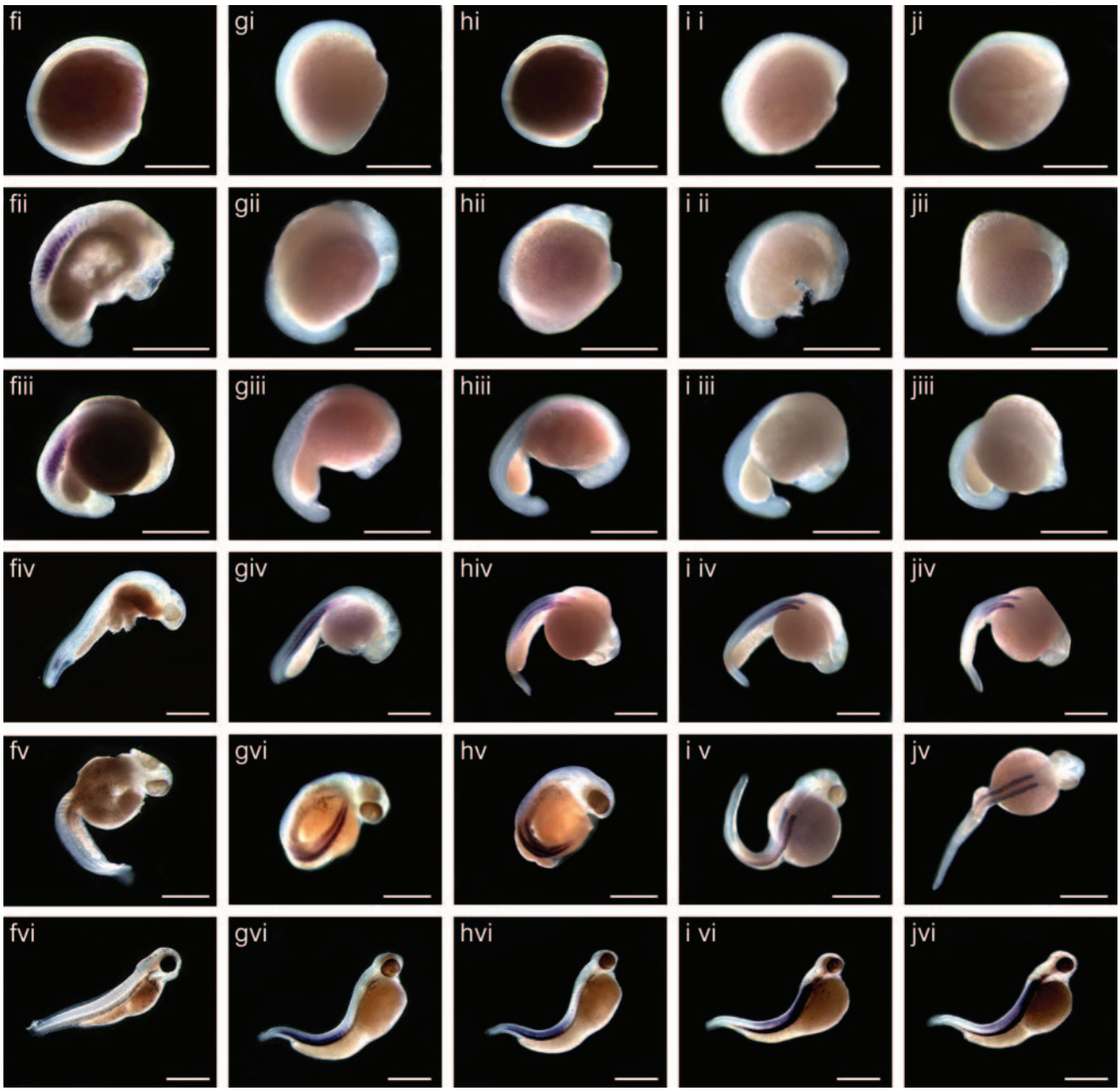

Fig. 1

22 h in the 18°C group, and 12 h in the 25°C group. Somites were formed at ~1 per hour (18°C) and ~2 per hour (25°C) to a final number of 38 or 39 (both groups). Time until 50% hatching was 120 h at 18°C and 55 h at 25°C. At hatching, embryos measured 3.72±0.39cm (S.D.), and there was no significant difference between temperature groups (Student's *t*-test, $P>0.05$, $N=20$ fish per group).

Expression of *MyoD* and *Myf-5* occurred simultaneously following epiboly in the pre-somitic mesoderm. *MyoD* was expressed in a pair of bilaterally symmetrical strips corresponding to the position of the adaxial cells (Fig. 1c,d), adjacent to the notochord. *Myf-5* was also expressed in the adaxial cells, but as development proceeded, transcripts spread further laterally into the mesoderm (Fig. 1a,b). Before the appearance of the first somite furrows, *Myf-5* expression could be seen very faintly in two bands corresponding to the cellular fields of the first somites (Fig. 2a). As soon as each somite formed, however, expression of *Myf-5* was downregulated. By contrast, expression of *MyoD* persisted as the somites were formed (Figs 1c,d). The dynamics of expression were such that at any time during somitogenesis, the newest ~12 somites stained positive for *MyoD* mRNA (Fig. 2b).

Expression of *myogenin* was switched on in the somites later than *Myf-5* and *MyoD* (Fig. 1e,f). The extent of staining lagged behind that of *MyoD* by ~5 somites, and ~12 were stained at any one time (Fig. 1c). The expression patterns of all three transcripts gave the appearance of a rostral–caudal wave, initiated by *Myf-5*, and followed by *MyoD* and *myogenin*, respectively (Fig 1a–f). No differences were seen between 18 and 25°C groups relative to developmental stage.

The embryonic forms of *MyHC*, *Eggs22* and *Eggs24* were first seen at the 25–30 somite stage, beginning in the anterior-most somites and progressing caudally (Fig 1g–j). After the completion of somitogenesis, *Eggs22* transcripts became concentrated in the caudal somites, whereas *Eggs24* predominantly stained the anterior somites. Expression persisted post-hatch, but was much reduced. No differences were seen between the 18 and 25°C groups with respect to developmental stage (Fig. 1g–j). No expression of mRNA for the 10°C-type, intermediate-type and 30°C-type MyHC isoforms were seen at any stage. Positive dot-blots using RNA isolated from fast muscle of 10 and 30°C acclimated adult carp (10 cm total length), alongside negative blots from the 18°C and 25°C incubated post-hatch larvae provided a positive control for the *in situ* results (Fig. 3).

The neighbour-joining tree separated the four MRFs in relation to the outgroup Ascidian sequence (Fig. 4; for accession numbers see Data 2 in supplementary material). Within genes, clades broadly reflected evolutionary relationships, and the majority of the bootstrap values were high (>90%). Further support was given by comparison with the tree from parsimony analysis, which was almost identical. Importantly, the *Xenopus MyoD* and *myogenin* paralogues clustered together, as did the trout *MyoD* paralogues. By contrast, the seabream amino acid sequences were more highly divergent.

Discussion

The expression patterns of carp *MyoD* and *myogenin* more-closely resembled those of the zebrafish than those of other teleosts studied to date. In the trout, *MyoD* expression extends laterally outwards from the adaxial cells relatively late in development, after the somites acquire their chevron shape, whereas in the other fish species studied (zebrafish, Weinberg et al., 1996; herring, Temple et al., 2001; seabream, Tan and Du, 2002), adaxial cell expression of *MyoD* occurs across the somites soon after their formation. Furthermore, expression of *myogenin* persisted for longer in the somites than expression of *MyoD*, unlike in the herring, where the reverse is the case (Temple et al., 2001). Expression of carp *Myf-5* also resembled that seen in the zebrafish, although in the zebrafish a more clearly defined banding pattern is seen in the presomitic mesoderm prior to somite formation, with at least five *Myf-5*-positive presomitic bands (Coutelle et al., 2001). Similarities between expression

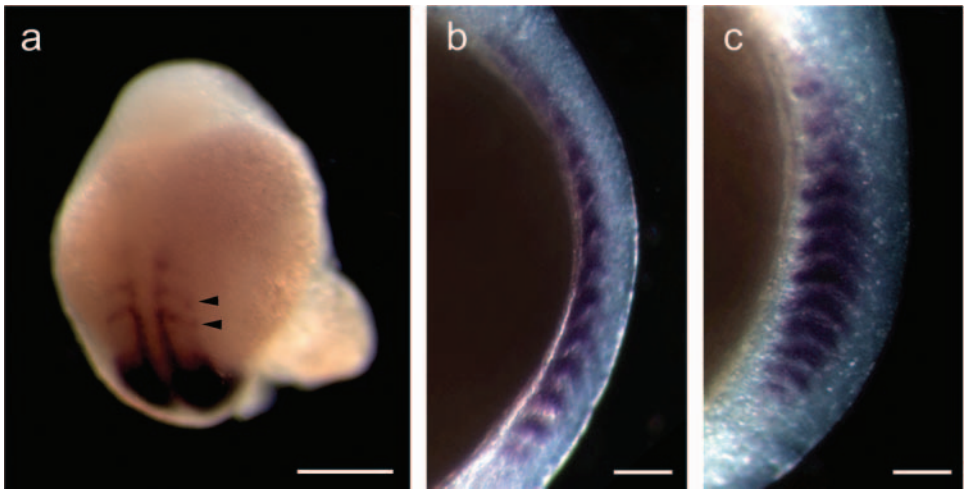

Fig. 2. (a) *Myf-5* expression in two presomitic bands (arrowheads) immediately prior to the onset of somitogenesis. Scale bar, 500 µm. (b) *MyoD* expression in the first ~12 somites. Scale bar, 100 µm. (c) *Myogenin* expression in ~12 somites (17-somite stage embryo). Scale bar, 100 µm.

patterns might be expected between the carp and zebrafish given that they are taxonomically closely related, both belonging to the family Cyprinidae. *MRF4* expression has not been studied in any teleost to date, although a genomic clone has been isolated from the pufferfish, *Fugu rubripes* (Carvajal et al., 2001; Fig. 4), and a cDNA sequence exists for the zebrafish (Fig. 4; see Data 1 in supplementary material).

The expression patterns of the genes encoding the embryonic MyHC isoforms (*Eggs22* and *Eggs24*) also showed no difference in timing between temperature groups, and the timing of transcription was broadly similar to that described by Ennion et al. (1999). However, the finding that the adult 10°C-type, intermediate-type and 30°C-type *MyHC*s were not expressed, even as the embryonic forms disappeared, was significant although not altogether unexpected. Other MyHC isoforms must be present to bridge the gap, either further embryonic forms, adult forms, or forms specific to the larval stages. Embryonic MyHC isoforms have been described in a variety of other species including human (Eller et al., 1989; Karsch-Mizrachi et al., 1989) rat (Strehler et al., 1986), chicken (Molina et al., 1987; Hofmann et al., 1988) and *Xenopus* (Radice and Malacinski, 1989). However, the myosin heavy chain multigene family in the carp is particularly large. Kikuchi et al. (1999) isolated 29 different genomic clones, more than twice the number present in humans (Soussi-Yanacostas et al., 1993; Kikuchi et al., 1999). Such diversity in carp myosin genes probably reflects the need for different molecular characteristics during the life cycle, as a result of allometric scaling relationships and temperature acclimation (Imai et al., 1997; Ennion et al., 1999; Kikuchi et al., 1999; Cole and Johnston, 2001).

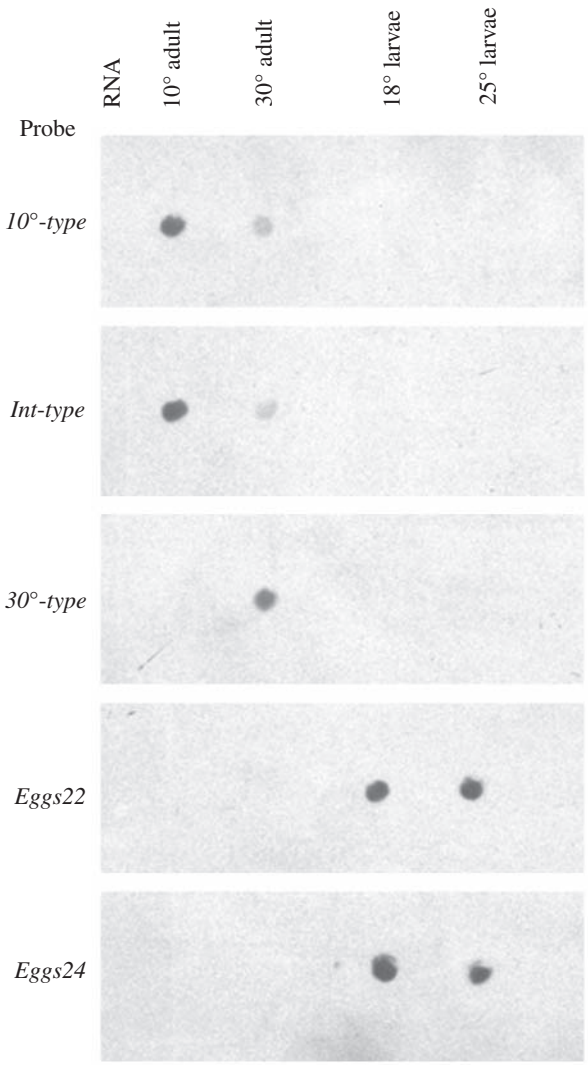

Fig. 3. RNA dot-blots showing expression of myosin heavy chain isoforms in larvae grown at 18 and 25°C, and in adult (10 cm) fish acclimated to 10 and 30°C.

The neighbour-joining tree for the MRF family is shown in Fig. 4. The topology supports the notion, proposed by Atchley et al. (1994), that all four members evolved from a common ancestor by gene duplication. After an initial duplication, each lineage divided again, one giving rise to *Myf-5* and *MyoD*, and the other giving rise to *myogenin* and *MRF4*. However, despite the fact that *MRF4* is most-closely related to *myogenin*, in the human and pufferfish the *MRF4* gene is most-closely associated spatially with *Myf-5*. In human, *MYF5* and *MRF4* are located on chromosome 12, with their start codons only 8.5 kb apart (Patapoutian et al., 1993) and in pufferfish they are even closer together, with their start codons differing by less than 5 kb (genomic clone encoding *Myf-5* and *MRF4*, NCBI accession no. AJ308546). It is possible that the functions of the two genes demand that they respond to the same control regions, or that their close proximity is essential for their autoregulation, a hypothesis that is supported by the fact that in all of the three *Mrf4*-knockout mice constructed, *Myf5* function is also affected (Summerbell et al., 2002).

Recently, the view of the MRFs as a discrete family of four transcription-factor-encoding genes has been clouded by the discovery of parologous forms, which have diverged in function in some species. Rescan and Gauvry (1996) isolated a second form of *MyoD* from the trout, and demonstrated different expression patterns using *in situ* hybridization. *MyoD1* was expressed in the adaxial cells of the unsegmented mesodermal plate and in the developing somites. *MyoD2* expression, however, was initiated later and was limited to the posterior compartment of the somite. Similarly, in *Xenopus*, paralogous forms of *MyoD* and *myogenin* have been isolated. One *MyoD* transcript (*xlmf25*) is expressed as a maternal mRNA in the early embryo, while the other (*xlmf1*) is activated from the zygotic genome near to the beginning of somitogenesis (Scales et al., 1990, 1991). Of the *myogenin* transcripts, one (*XmyogU2*) is expressed during embryogenesis, while the other (*XmyogU1*) is exclusive to the adult skeletal muscle (Charbonnier et al., 2002).

The expression of parologous genes is common in some organisms, such as trout and *Xenopus*, both of which have undergone recent genome duplication events and are in a state of pseudotetraploidy (Allendorf and Thorgaard, 1984; Hughes and Hughes, 1993; Rescan, 2001). However, the non-tetraploid gilthead seabream also differentially expresses two parologous

forms of *MyoD* (Tan and Du, 2002). In this case, the sequence identity of the two forms is lower than for the tetraploid organisms (Fig. 4), suggesting a more-ancient duplication event. Interestingly, a cDNA that clustered with seabream *MyoD2* (Fig. 3) was recently isolated from the Atlantic cod (Hall et al., 2003).

No parologous forms of MRF family genes have been isolated from any of the tetrapod lineage, with the exception of the tetraploid *Xenopus*, and, paradoxically, despite the availability of whole genome shotgun sequences, in the zebrafish or pufferfish. The dynamics of teleost genome evolution is extremely complex, with evidence for specific genome duplication events remaining a contentious issue (Meyer and Malaga-Trillo, 1999; Meyer and Schartl, 1999; Robinson-Rechavi et al., 2001a,b; Taylor et al., 2001a,b). In any case, whether at the whole-genome or more-regional level, teleost genomes are characterised by a high rate of duplication followed by substantial gene loss (Robinson-Rechavi et al., 2001c; Sibthorpe, 2002; Smith et al., 2002). Further characterizing the molecular evolution of the MRF family in relation to function remains a challenging, but potentially rewarding, task.

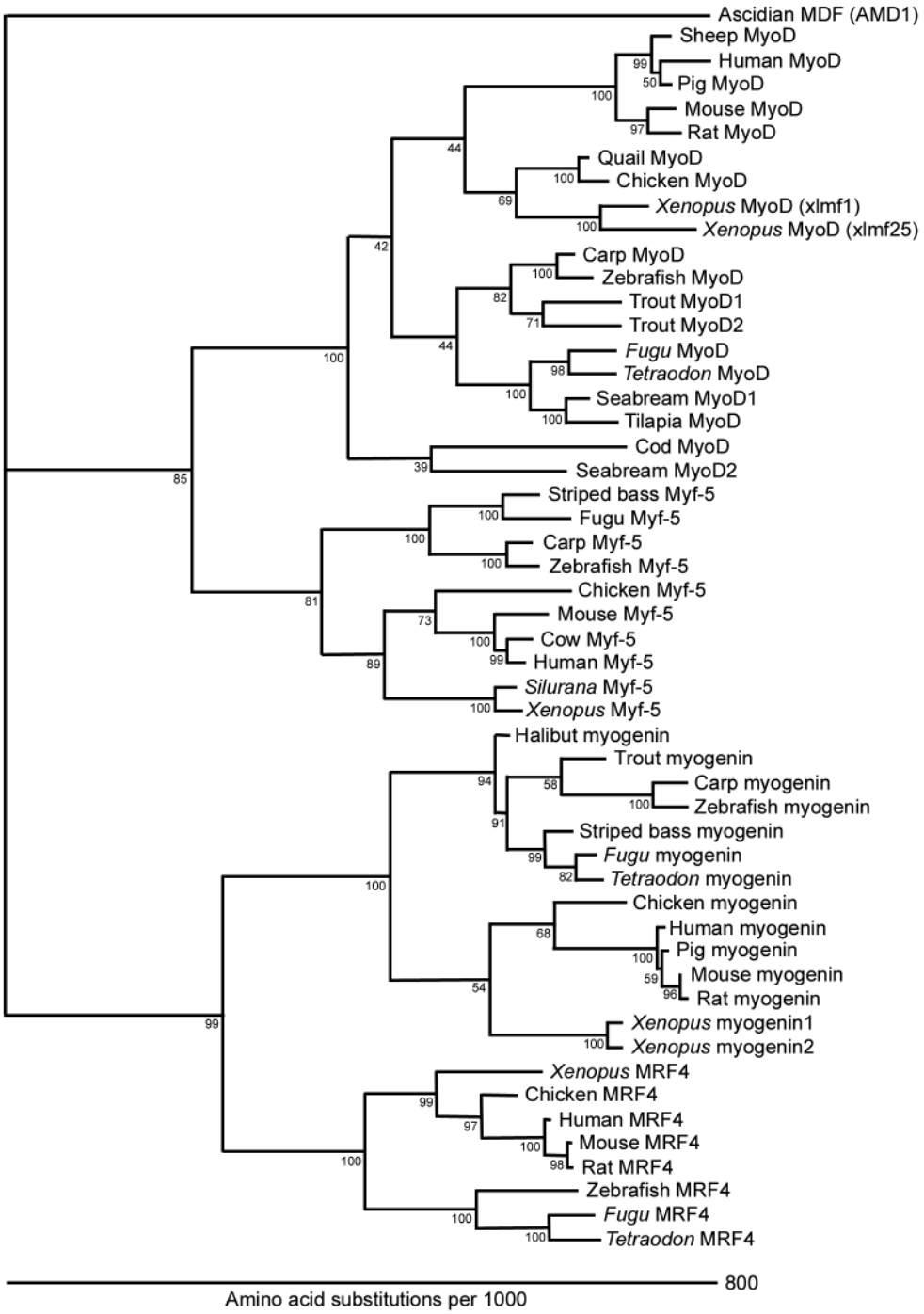

Fig. 4. Neighbour-joining tree of vertebrate myogenic regulatory factors with an Ascidian outgroup. Node numbers refer to the percentage of 1000 bootstrap pseudoreplicates supporting a clade. Branch lengths are proportional to the number of amino acid substitutions.

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Note added in proof

Since going to press, important new evidence has arisen regarding genome duplication in the teleost lineage. Jaillon et al. (2004) present near definitive evidence from *Tetraodon nigroviridis* of an ancient full-scale genome duplication. They demonstrate firstly, that every chromosome was involved in large-scale duplication, and secondly, a striking pattern of double synteny, with one chromosomal region in humans matching two in the pufferfish, across the whole genome.

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Fugu MyoD ORF predicted from Ensembl (release 23.2c.1) genomic Scaffold 1617, bases 21499-23256

```
1          ATGGAGCTGTCGGAGATCTCCTTCTCCATCCCTGCCGCTGACGACTTCTATGACGACCCC
1           M  E  L  S  E  I  S  F  S  I  P  A  A  D  D  F  Y  D  D  P

61         TGCTTCAGCACCTCTGACATGCACTTTTTTGAGGACATGGACCCCCGCCTGGTCCACGCG
21          C  F  S  T  S  D  M  H  F  F  E  D  M  D  P  R  L  V  H  A

121        GGCCTGCTGAAGCCGGATGACTGCTGCTCTTCATCCTCACTCTCTCCTTCCTCATCTTCC
41          G  L  L  K  P  D  D  C  C  S  S  S  S  L  S  P  S  S  S  S

181        GCTTCCCCATCGTCCCTTCTGCACATTCATCATCACACCGAGGCGGAGGACGACGAGCAC
61          A  S  P  S  S  L  L  H  I  H  H  H  T  E  A  E  D  D  E  H

241        ATCCGTGCACCCAGCGGGCACCACCATGCAGGCCGCTGTCTCCTCTGGGCCTGCAAGGCC
81          I  R  A  P  S  G  H  H  H  A  G  R  C  L  L  W  A  C  K  A

301        TGCAAGCGGAAGACTACAAACGTGGACCGACGGAAGGCGGCGACGCTGCGTGAGCGGCGG
101         C  K  R  K  T  T  N  V  D  R  R  K  A  A  T  L  R  E  R  R

361        CGGCTAAGCAAAGTCAACGAGGCCTTCGAGACGTTGAAACGCTGCACAAACACCAACCCG
121         R  L  S  K  V  N  E  A  F  E  T  L  K  R  C  T  N  T  N  P

421        AACCAGCGGCTGCCCAAAGTGGAGATCCTGAGGAACGCCATCAGCTATATCGAATCCCTG
141         N  Q  R  L  P  K  V  E  I  L  R  N  A  I  S  Y  I  E  S  L

481        CAGGCACTGCTCCGAGGCGGCCAGGACGAGGCCTTCTACACCGTTCTGGAGCACTACAGC
161         Q  A  L  L  R  G  G  Q  D  E  A  F  Y  T  V  L  E  H  Y  S

541        GGGGACTCCGACGCGTCTAGCCCTCGCTCCAACTGCTCCGATGGCATGACGGATTTTAAC
181         G  D  S  D  A  S  S  P  R  S  N  C  S  D  G  M  T  D  F  N

601        GGTCCGACCTGTCAATCAAACAGAAGAGGAAGCTATTACAGCAGCTATTTCTCGCAAACT
201         G  P  T  C  Q  S  N  R  R  G  S  Y  Y  S  S  Y  F  S  Q  T

661        CCAAAAAGCCTGAAGGCGGAGCGGAACTCCAGTCTCGACTGTCTGTCCAGCATCGTGGAG
221         P  K  S  L  K  A  E  R  N  S  S  L  D  C  L  S  S  I  V  E

721        CGGATCTCCACCGCCACCAGCAGCGGGCCGCCACCCGTAGACGGCCGCGGGTCTCCTGGG
241         R  I  S  T  A  T  S  S  G  P  P  P  V  D  G  R  G  S  P  G

781        CCCCTTCAGGCCTCCTCTCCAAGGAGCAGTCGGGAACCAAACCTGATTTATCAGGTCCTG
261         P  L  Q  A  S  S  P  R  S  S  R  E  P  N  L  I  Y  Q  V  L
```

Fugu myogenin ORF predicted from Ensembl (release 23.2c.1) genomic Scaffold 208, bases 195095-196347

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1          ATGGAGCTTTTTGAGACCAACCCTTACTTCTTTCCCGACCAGCGCTTCTACGAGGGAGGA
1           M  E  L  F  E  T  N  P  Y  F  F  P  D  Q  R  F  Y  E  G  G

61         GATACCTACTTCCCCTCTCGTTTACCTGGGTCCTACGACCAAGGCACCTACCAGGATAGG
21          D  T  Y  F  P  S  R  L  P  G  S  Y  D  Q  G  T  Y  Q  D  R

121        AACACCATGATGGGCTTGTGTGGGAGTCTGTCCGGAGGTGTGGATGTTGGAGTGACAGGG
41          N  T  M  M  G  L  C  G  S  L  S  G  G  V  D  V  G  V  T  G

181        ACAGAGGACAAAGCCTCTCCATCCAGCCTGTCACCTCACTCTGAGCCACACTGCCCGGGC
61          T  E  D  K  A  S  P  S  S  L  S  P  H  S  E  P  H  C  P  G

241        CAGTGCCTTCCCTGGGCCTGCAAGTTATGCAAGAGGAAGACGGTCACCATGGACCGCCGG
81          Q  C  L  P  W  A  C  K  L  C  K  R  K  T  V  T  M  D  R  R

301        AGAGCGGCCACGCTGAGAGAGAAGAGGCGCCTGAAGAAGGTGAACGAGGCCTTCGACGCT
101         R  A  A  T  L  R  E  K  R  R  L  K  K  V  N  E  A  F  D  A

361        TTGAAGAGGAGCACGTTGATGAACCCAAACCAGAGGCTGCCCAAGGTGGAGATCCTCAGG
121         L  K  R  S  T  L  M  N  P  N  Q  R  L  P  K  V  E  I  L  R

421        AGCGCCATCCAGTATATCGAAAAGCTACAGGCCTTGGTGTCCTCCCTCAACCAGCAGGAC
141         S  A  I  Q  Y  I  E  K  L  Q  A  L  V  S  S  L  N  Q  Q  D

481        ACTGAGACGGGACAGCAGGGACTGCACTTCCGGACCAGCGCGGTCCAACCCAGGGTGTCG
161         T  E  T  G  Q  Q  G  L  H  F  R  T  S  A  V  Q  P  R  V  S

541        TCATCCAGCGAGCCCAGCTCAGGCAGCACGTGCTGCAGCAGCCCAGAGTGGAGCAGCACC
181         S  S  S  E  P  S  S  G  S  T  C  C  S  S  P  E  W  S  S  T

601        CCCGACCAGTGCACGCAGAGCTACAGCAGCGAGGATCTTCTGAGCGCTGCCGACTCTCCG
201         P  D  Q  C  T  Q  S  Y  S  S  E  D  L  L  S  A  A  D  S  P

661        GACCAAGGGAGCATGCGCACCCTGACCGCCATCGTGGACAGCATCTCTGCAGCGGACGCC
221         D  Q  G  S  M  R  T  L  T  A  I  V  D  S  I  S  A  A  D  A

721        GCCGTGGCCTTTTCTATGGACATTCCCAAG
241         A  V  A  F  S  M  D  I  P  K
```

Tetraodon MyoD ORF predicted from Genoscope (release 6) genomic scaffold SCAF7217, bases 4227-5573

```
1          ATGGAGCTCTCGGAGATCTCCTTCTCCATCCCAGCCGCTGATGACTTCTATGACGACCCC
1           M  E  L  S  E  I  S  F  S  I  P  A  A  D  D  F  Y  D  D  P

61         TGTTTCAGCACCTCTGACATGCACTTTTTTGAGGACCTGGACCCCCGCCTTGTCCACACG
21          C  F  S  T  S  D  M  H  F  F  E  D  L  D  P  R  L  V  H  T

121        AGCCTGCTGAAGCCAGATGACTGTTGCTCCTCATCCTCACTCTCCCCTTCGTCTTCTTAC
41          S  L  L  K  P  D  D  C  C  S  S  S  S  L  S  P  S  S  S  Y

181        TCGTCTCCATCCTCCCTCCAGCACCACCATCACCACGCTGAAGCGGAGGACGACGACGAC
61          S  S  P  S  S  L  Q  H  H  H  H  H  A  E  A  E  D  D  D  D

241        GTCCGTGCACCCAGCGGGCACCACCAGGCGGGTCGCTGCCTCCTCTGGGCCTGCAAGGCC
81          V  R  A  P  S  G  H  H  Q  A  G  R  C  L  L  W  A  C  K  A

301        TGCAAACGGAAGACCAACGCGGACCGGCGGAAGGCGGCGACGCTGCGTGAGCGGCGGCGC
101         C  K  R  K  T  N  A  D  R  R  K  A  A  T  L  R  E  R  R  R

361        CTCAGCAAAGTCAACGAGGCCTTCGAGACCCTGAAGCGCTGCACCAGCGCCAACCCCAAC
121         L  S  K  V  N  E  A  F  E  T  L  K  R  C  T  S  A  N  P  N

421        CAGCGGCTGCCCAAAGTGGAGATCCTGAGGAACGCCATCAGCTACATCGAGTCCCTGCAG
141         Q  R  L  P  K  V  E  I  L  R  N  A  I  S  Y  I  E  S  L  Q

481        GCGCTGCTCCGAGGCGGCCAGGACGAGGCCTTCTACCCTGTGCTGGAGCACTACAGCGGG
161         A  L  L  R  G  G  Q  D  E  A  F  Y  P  V  L  E  H  Y  S  G

541        GAGTCGGACGCGTCCAGCCCCCGCTCCAACTGCTCCGACGGCATGACGGATTTTAATGGT
181         E  S  D  A  S  S  P  R  S  N  C  S  D  G  M  T  D  F  N  G

601        CCTACCTGTCAATCAAGCAGAAGAGGAAGTTATGACAGCAGCTATCTGTCACAAACTCCA
201         P  T  C  Q  S  S  R  R  G  S  Y  D  S  S  Y  L  S  Q  T  P

661        CTGAAGGCGGAGCGCAACTCCAGTCTGGACTGTCTGTCCAGCATCGTGGAGCGGATCTCC
221         L  K  A  E  R  N  S  S  L  D  C  L  S  S  I  V  E  R  I  S

721        ACGGACACCAGCAGCGGTGTNCCGCACCCCGCAGAGGGTCCGCGCCACCCCGGGTGTCCC
241         T  D  T  S  S  G  V  P  H  P  A  E  G  P  R  H  P  G  C  P

781        GTCCTGGCCACCCCCCCGCAGAGCAGCCGGGACCCAAACCTG
261         V  L  A  T  P  P  Q  S  S  R  D  P  N  L
```

Tetraodon myogenin ORF predicted from Genoscope (release 6) genomic scaffold SCAF14528, bases 234783-235947

```
1          ATGGAGCTTTTTGAGACCAACCCTTACTTCTTTCCTGACCAGCGCTTCTACGAGGGAGGA
1           M   E   L   F   E   T   N   P   Y   F   F   P   D   Q   R   F   Y   E   G   G

61         GATAGCTACTTCCCCTCTCGCCTACCGGGGTCCTACGACCAAAGCACCTACCAGGACCGG
21          D   S   Y   F   P   S   R   L   P   G   S   Y   D   Q   S   T   Y   Q   D   R

121        AACTCCATGATGGGCTTGTGCGGGAGTCTGTCTGGAGGTGTCGACGTTGGAGTGACAGGG
41          N   S   M   M   G   L   C   G   S   L   S   G   G   V   D   V   G   V   T   G

181        ACAGAGGACAAAGCCTCTCCGTCCAGCCTGTCACCTCACTCTGAGCCACACTGCCCGGGT
61          T   E   D   K   A   S   P   S   S   L   S   P   H   S   E   P   H   C   P   G

241        CAGTGCCTTCCCTGGGCCTGCAAGATATGCAAGAGGAAGACGGTCACCATGGACCGTCGG
81          Q   C   L   P   W   A   C   K   I   C   K   R   K   T   V   T   M   D   R   R

301        AGGGCCGCCACGCTGAGGGAGAAGAGGCGCCTGAAGAAGGTGAACGAGGCCTTCGACGCT
101         R   A   A   T   L   R   E   K   R   R   L   K   K   V   N   E   A   F   D   A

361        TTGAAGAGGAGCACGTTGATGAACCCCAACCAGAGGCTGCCCAAGGTGGAGATCCTCAGG
121         L   K   R   S   T   L   M   N   P   N   Q   R   L   P   K   V   E   I   L   R

421        AGCGCCATCCAGTACATCGAAAGGCTGCAGGCCTTGGTGTCCTCCCTCAACCAGCAGGAC
141         S   A   I   Q   Y   I   E   R   L   Q   A   L   V   S   S   L   N   Q   Q   D

481        ACTGAGACGGCGCAGCAGGCGCTGCACTTCCGGACCAGCGCGGCCCAACCCAGAGTGTCG
161         T   E   T   A   Q   Q   A   L   H   F   R   T   S   A   A   Q   P   R   V   S

541        TCATCCAGCGAGCCCAGCTCAGGCAGCACCTGCTGCAGCAGCCCAGAGTGGAGCAGCACC
181         S   S   S   E   P   S   S   G   S   T   C   C   S   S   P   E   W   S   S   T

601        CCTGAACAGTGCACGCAGAGCTACAGCAGCGAGGATCTTCTGAGTGCTGCCGACTCTCCG
201         P   E   Q   C   T   Q   S   Y   S   S   E   D   L   L   S   A   A   D   S   P

661        GAGCAGGGGAGCATGCGTACCCTGACCGCCATCGTGGACAGCATCTCTGCAGCGGACGCC
221         E   Q   G   S   M   R   T   L   T   A   I   V   D   S   I   S   A   A   D   A

721        GCCGTGGCCTTT
241         A   V   A   F
```

Tetraodon MRF4 ORF prediction from Genoscope (release 6) genomic scaffold SCAF14691, bases 236320-237613

```
1          ATGATGGACCTTTTTGAGACCAACACTTATCTTTTCAATGATTTGCGCTATCTGGAGGAG
1           M  M  D  L  F  E  T  N  T  Y  L  F  N  D  L  R  Y  L  E  E

61         GGGGATCATGGACCACTGCAGCACTTGGACATGTCCGGGGTGTCCCCCCTCTATGACGGG
21          G  D  H  G  P  L  Q  H  L  D  M  S  G  V  S  P  L  Y  D  G

121        AACCACAGCCCGCTGTCTCCGGGTCCGGACAACGTCCCCTCTGAGACCGGGGGCGAGAGC
41          N  H  S  P  L  S  P  G  P  D  N  V  P  S  E  T  G  G  E  S

181        AGCGGGGACGAACACGTCCTGGCGCCGCCGGGGGTGCGCGCCCACTGCGAGGGCCAGTGT
61          S  G  D  E  H  V  L  A  P  P  G  V  R  A  H  C  E  G  Q  C

241        CTCATGTGGGCCTGCAAGGTCTGCAAGCGCAAGTCGGCGCCCACCGACCGGCGCAAGGCC
81          L  M  W  A  C  K  V  C  K  R  K  S  A  P  T  D  R  R  K  A

301        GCCACGCTGCGGGAGAGGAGGAGGCTGAAGAAGATCAACGAGGCCTTCGACGCGCTCAAG
101         A  T  L  R  E  R  R  R  L  K  K  I  N  E  A  F  D  A  L  K

361        AGGAAGAGCGTGGCCAACCCCAACCAGAGGCTGCCCAAGGTGGAGATCCTGCGCAGCGCC
121         R  K  S  V  A  N  P  N  Q  R  L  P  K  V  E  I  L  R  S  A

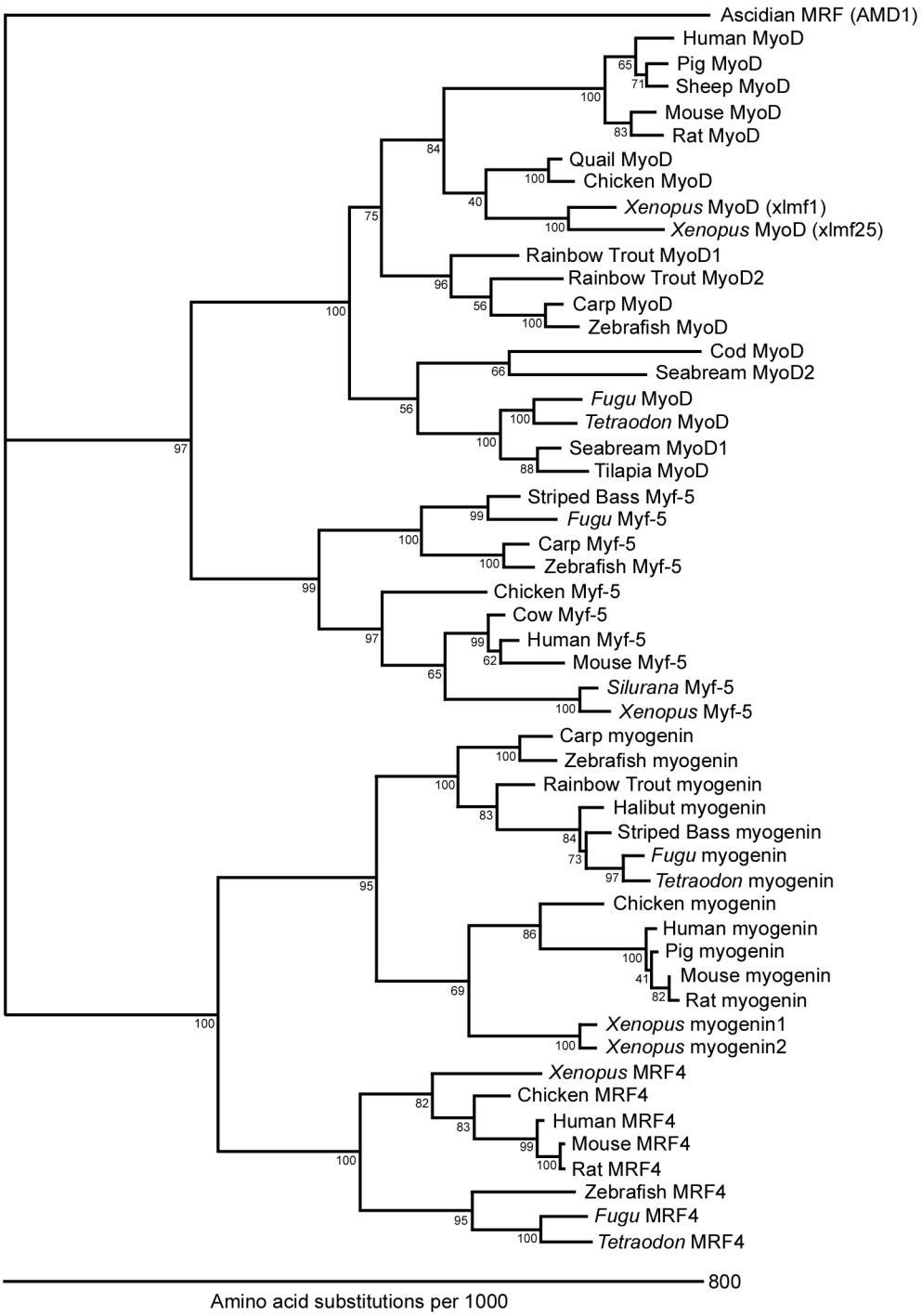
421        ATCAGCTACATCGAGCGGCTGCAGGAGCTGCTGCAGAGCCTGGACGAGCAGGAGCGCAGC
141         I  S  Y  I  E  R  L  Q  E  L  L  Q  S  L  D  E  Q  E  R  S

481        CCGAAGGGAGCCGGCGACGGCCCAGGAGAAGTTCCACAGCGACCCGGCGGCGGCGGCGAC
161         P  K  G  A  G  D  G  P  G  E  V  P  Q  R  P  G  G  G  G  D

541        TACTGCTGGAAAAAGGCCTCGGAGACGTGGCCGACCTCCGCCGACCATTCCGCCATCATT
181         Y  C  W  K  K  A  S  E  T  W  P  T  S  A  D  H  S  A  I  I

601        AACCAGAGAGACGGAGCCTGCGAGTCTTCGGCCTCCTCCAGCCTCCTCTGCCTCTCCTCC
201         N  Q  R  D  G  A  C  E  S  S  A  S  S  S  L  L  C  L  S  S

661        ATCGTCAGCAGCATCAGCGACGACAAGACGGACCTCAGACACAGCGTCCCGGGGAAC
221         I  V  S  S  I  S  D  D  K  T  D  L  R  H  S  V  P  G  N
```


Ascidian MRF (AMD1)
Human MyoD
Pig MyoD
Sheep MyoD
Mouse MyoD
Rat MyoD
Quail MyoD
Chicken MyoD
Xenopus MyoD (xlmf1)
Xenopus MyoD (xlmf25)
Rainbow Trout MyoD1
Rainbow Trout MyoD2
Carp MyoD
Zebrafish MyoD
Cod MyoD
Seabream MyoD2
Fugu MyoD
Tetraodon MyoD
Seabream MyoD1
Tilapia MyoD
Striped Bass Myf-5
Fugu Myf-5
Carp Myf-5
Zebrafish Myf-5
Chicken Myf-5
Cow Myf-5
Human Myf-5
Mouse Myf-5
Silurana Myf-5
Xenopus Myf-5
Carp myogenin
Zebrafish myogenin
Rainbow Trout myogenin
Halibut myogenin
Striped Bass myogenin
Fugu myogenin
Tetraodon myogenin
Chicken myogenin
Human myogenin
Pig myogenin
Mouse myogenin
Rat myogenin
Xenopus myogenin1
Xenopus myogenin2
Xenopus MRF4
Chicken MRF4
Human MRF4
Mouse MRF4
Rat MRF4
Zebrafish MRF4
Fugu MRF4
Tetraodon MRF4
800
Amino acid substitutions per 1000

Accession numbers for MRF sequences used in phylogenetic analyses

Ascidian MRF (AMD1)	D13507
Carp MyoD	AB012882
Chicken MyoD	L34006
Cod MyoD	AF329903
Fugu MyoD	Scaffold 1617, 21499-23259 (Ensembl)
Human MyoD	NM_002478
Mouse MyoD	XM_124916
Pig MyoD	U12574
Quail MyoD	L16686
Rainbow Trout MyoD1	X75798
Rainbow Trout MyoD2	Z46924
Rat MyoD	M84176
Seabream MyoD1	AF478568
Seabream MyoD2	AF478569
Sheep MyoD	X62102
Tetraodon MyoD	SCAF14528, 234783-235946 (Genoscope)
Tilapia MyoD	AF270790
Xenopus MyoD (xlmf1)	M31116
Xenopus MyoD (xlmf25)	M31118
Zebrafish MyoD	NM_131262
Carp Myf-5	AB012883
Chicken Myf-5	X73250
Cow Myf-5	M95684
Fugu Myf-5	AJ308546
Human Myf-5	NM_005593
Mouse Myf-5	XM_192677
Silurana Myf-5	AY050251
Striped Bass Myf-5	AF463525
Xenopus Myf-5	X56738
Zebrafish Myf-5	NM_131576
Carp myogenin	AB012881
Chicken myogenin	D90157
Fugu myogenin	Scaffold 208, 195095-196350 (Ensembl)
Halibut myogenin	AJ487982
Human myogenin	NM_002479
Mouse myogenin	D90156
Pig myogenin	U14331
Rainbow trout myogenin	Z46912
Rat myogenin	NM_017115
Tetraodon myogenin	SCAF14528, 234783-235946 (Genoscope)
Striped Bass myogenin	AF463526
Xenopus myogenin U1	AY046531
Xenopus myogenin U2	AY046532
Zebrafish myogenin	NM_131006
Chicken MRF4	D10599
Human MRF4	NM_002469
Mouse MRF4	NM_008657
Fugu MRF4	AJ308546
Rat MRF4	NM_013172
Tetraodon MRF4	SCAF14691, 236320-237612 (Genoscope)
Xenopus MRF4	S84990
Zebrafish MRF4	AY_335193