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# The myosin light chain 1 isoform associated with masticatory myosin heavy chain in mammals and reptiles is embryonic/atrial MLC1

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## SUMMARY

**We recently reported that masticatory myosin heavy chain (MHC-M) is expressed as the exclusive or predominant MHC isoform in masseter and temporalis muscles of several rodent species, contrary to the prevailing dogma that rodents express almost exclusively MHC isoforms that are typically found in fast limb muscles and not masticatory myosin. We also reported that the same rodent species express the embryonic/atrial isoform of myosin light chain 1 (MLC1E/A) in jaw-closing muscles and not a unique masticatory MLC1 isoform that others have reported as being expressed in jaw-closing muscles of carnivores that express MHC-M. The objective of this study was to test the hypothesis that MLC1E/A is consistently expressed in jaw-closing muscles whenever MHC-M is expressed as the predominant or exclusive MHC isoform. Jaw-closing muscles, fast and slow limb muscles, and cardiac atria and ventricles of 19 species (six Carnivora species, one Primates species, one Chiroptera species, five marsupial species, an alligator and five turtle species) were analyzed using protein gel electrophoresis, immunoblotting, mass spectrometry and RNA sequencing. Gel electrophoresis and immunoblotting indicate that MHC-M is the exclusive or predominant MHC isoform in the jaw-closing muscles of each of the studied species. The results from all of the approaches collectively show that MLC1E/A is exclusively or predominantly expressed in jaw-closing muscles of the same species. We conclude that MLC1E/A is the exclusive or predominant MLC1 isoform that is expressed in jaw-closing muscles of vertebrates that express MHC-M, and that a unique masticatory isoform of MLC1 probably does not exist.**

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/213/10/1633/DC1

Key words: myosin light chain, masticatory myosin, jaw-closing muscles.

## INTRODUCTION

We recently reported that masticatory myosin is the predominantly expressed myosin in jaw-closing muscles of several rodent species, all being members of the Sciuridae family. Masticatory myosin in Sciuridae consists of MHC-M, the masticatory isoform of myosin light chain 2 (MLC2M) and MLC1E/A (Reiser et al., 2009). Our report was the first description of masticatory myosin in any rodent species and the first report of MLC1E/A being associated with MHC-M. The initial discovery of masticatory myosin, in cat temporalis muscle, was reported by Rowlerson and co-workers (Rowlerson et al., 1981), who referred to it as 'superfast' myosin, and fibers expressing this myosin as 'superfast' fibers. The term, superfast, was based upon the observation that the myosin extracted from adult cat temporalis muscle hydrolyzes ATP at a rate that is two to three times greater than the myosin isolated from cat fast limb muscle. Masticatory myosin expression has since been described in additional members of Carnivora, as well as several other mammalian orders (Primates, Chiroptera, Didelphimorphia, Dasyuromorphia, Diprotodontia), reptiles (Crocodylia, Testudines) and fish (Pleurotremata) (reviewed by Hoh, 2002; Hoh et al., 2006). Masticatory myosin is expressed in jaw-closing muscles, as well as the tensor tympani, and the tensor veli palatini in at least some of the same species (Rowlerson et al., 1983b). This myosin has never been reported to be expressed in any limb, trunk or cardiac muscle. Another MHC isoform, referred to as extraocular MHC or laryngeal MHC (same protein), is expressed in extraocular and laryngeal muscles in some species. Extraocular/laryngeal MHC has been referred to as 'superfast' myosin in some reports, but this is a different protein, encoded by a different gene, and the label was intended to refer to the presumed high velocity of shortening in extraocular and laryngeal muscle fibers in which it is expressed (Briggs and Schachat, 2000; Shiotani and Flint, 1998) and knowing that these muscles can generate force at relatively high rates (e.g. Cooper and Eccles, 1930; Brown and Harvey, 1941; Bach-y-Rita and Ito, 1966; Martensson and Skoglund, 1964; Hall-Craggs, 1968).

The original description of masticatory (superfast) myosin (Rowlerson et al., 1981) referred to the MLC1 and MLC2 isoforms in cat temporalis as 'masticatory' isoforms. This was based upon the electrophoretic mobilities of MLC1 and MLC2 in cat temporalis being different from that of MLC1 and MLC2 in cat fast (MLC1F and MLC2F) and slow (MLC1S and MLC2S) limb muscles. MHC-M and MLC2M have been cloned and sequenced (Qin et al., 1994; Qin et al., 2002), and are, therefore, known to be unique isoforms. However, no direct evidence for the expression of a unique masticatory isoform of MLC1 has ever been reported. The primary objective of this study was to test if MLC1E/A is expressed with MHC-M in species other than those in the Sciuridae (Reiser et al., 2009). We identified the MLC1 isoform in jaw-closing muscles of species in which MHC-M was previously reported to be expressed by others (domestic cat, domestic dog, long-tailed macaque, Virginia opossum, gray short-tail opossum) (Rowlerson et al., 1981; Rowlerson et al., 1983b; Hoh et al., 1988; Sciote et al., 1995; Sciote and Rowlerson, 1998) and in fourteen additional species in which we found, and report for the first time, MHC-M expression, as being MLC1E/A.

## MATERIALS AND METHODS

### Samples

Samples were obtained from nineteen species of fourteen families in eight orders of two vertebrate classes (Table 1). The care, use and killing of the research animals at the Ohio State University from which samples were obtained for this project (domestic cat, domestic dog, long-tailed macaque, gray short-tail opossum) were in accordance with protocols approved by the Institutional Animal Care and Use Committee. Samples from free-living animals were (1) obtained from a local wildlife control company following culling, which was conducted in accordance with company policies and with the recommendations of the American Veterinary Medical Association Panel on Euthanasia (raccoon, skunk, Virginia opossum), (2) captured (turtles and bat), or (3) donated by a local trapper immediately after culling (gunshot), which was performed in accordance with state regulations (coyote). Additional samples were obtained from animals that were euthanized or had very recently died (within a few hours) at the Columbus Zoo and Aquarium (lion, tiger quoll, feathertail glider, sugar glider) and the Louisiana Department of Wildlife and Fisheries Rockefeller Wildlife Refuge (American alligator). All animals were believed to be adults, based upon body mass or actual birth data. Samples were obtained from only one animal of several species. Samples were obtained from two adults of domestic dog, domestic cat, raccoon, gray short-tailed opossum, Virginia opossum, skunk, bat, painted turtle and snapping turtle. Consistent results were obtained when samples from two animals of the same species were examined. The rationale for species selection for this study was based upon previous reports of masticatory myosin expression in specific species (Rowlerson et al., 1981; Rowlerson et al., 1983a; Rowlerson et al., 1983b; Sciote et al., 1995; Sciote and Rowlerson, 1998; Hoh 2002; Hoh et al., 2006) or phylogenetic relationships with species reported to express masticatory myosin.

The jaw-closing muscles that were sampled from all of the mammalian species were the masseter and/or temporalis. The fast-twitch tibialis cranialis was used as a source of fast myosin in every mammalian species except the sugar glider, in which the gastrocnemius was sampled. The digastric in the lion and the pectoralis in the bat were also sampled as sources of fast myosin. The soleus was sampled as a source of slow myosin in every mammalian species except the dog, coyote and sugar glider in which the deep portion of the gastrocnemius (a synergist of the slow soleus)

Table 1. Species studied

| Class | Order | Family | Common name/species |
|---|---|---|---|
| Mammalia (eutherian) | Carnivora | Felidae | Domestic cat (*Felis catus* Linnaeus 1758) |
| | Carnivora | Felidae | Lion (*Panthera leo* Linnaeus 1758) |
| | Carnivora | Canidae | Domestic dog (*Canis lupis familiaris* Linnaeus 1758) |
| | Carnivora | Canidae | Coyote (*Canis latrans* Say 1823) |
| | Carnivora | Mephitidae | Striped skunk (*Mephitis mephitis* Schreber 1776) |
| | Carnivora | Procyonidae | Northern raccoon (*Procyon lotor* Linnaeus 1758) |
| | Primates | Cercopithecidae | Long-tailed macaque (*Macaca fascicularis* Raffles 1821) |
| | Chiroptera | Vespertilionidae | Big brown bat (*Eptesicus fuscus* Palisot de Beauvois 1796) |
| Mammalia (marsupial) | Didelphimorphia | Didelphidae | Gray short-tailed opossum (*Monodelphis domestica* Wagner 1842) |
| | Didelphimorphia | Didelphidae | Virginia opossum (*Didelphis virginiana* Kerr 1792) |
| | Dasyuromorphia | Dasyuridae | Tiger quoll (*Dasyurus maculatus* Kerr 1792) |
| | Diprotodontia | Acrobatidae | Feathertail glider (*Acrobates pygmaeus* Shaw 1793) |
| | Diprotodontia | Petauridae | Sugar glider (*Petaurus breviceps* Waterhouse 1839) |
| Reptilia | Crocodilia | Crocodylidae | American alligator (*Alligator mississippiensis* Daudin 1801) |
| | Testudines | Chelydridae | Common snapping turtle (*Chelydra serpentina* Linnaeus 1758) |
| | Testudines | Trionychidae | Spiny softshell turtle (*Apalone spinifera* Lesueur 1827) |
| | Testudines | Emydidae | Painted turtle (*Chrysemys picta* Schneider 1783) |
| | Testudines | Emydidae | Common map turtle (*Graptemys geographica* Le Sueur 1817) |
| | Testudines | Emydidae | Red-eared slider (*Trachemys scripta elegans* Wied-Neuwied 1839) |

was sampled. The soleus muscle was carefully sampled in gray short-tailed opossum and Virginia opossum in which the soleus is fused with the gastrocnemius muscle along much of its length (Stein, 1981; Peters et al., 1984).

The jaw-closing muscles of the alligator that were sampled were the adductor mandibulae externus superficialis (separate samples were prepared from the red, white and deep portions and a suborbital region) and the adductor mandibulae posterior [illustrated in Holliday and Witmer (Holliday and Witmer, 2007)]. The soleus and tibialis cranialis from one hindlimb were selected as alligator slow and fast leg muscles, respectively. The large pars profunda of the external adductor [illustrated in figure 3a of Lemell et al. (Lemell et al., 2000)] was selected as a jaw-closing muscle in all five turtle species. The coracohyoideus was selected as a jaw-opener in turtles (Lemell et al., 2000). The flexor digitorum longus, with ~50% of the fibers reported to be slow, and the fourth head of the testo-cervicis, with many slow tonic fibers (Callister et al., 2005), were studied in each turtle species, as well. The iliofibularis, with predominantly fast fibers (Callister et al., 2005), was also sampled in the red-eared slider.

Atrial and ventricular samples were obtained from every mammalian and reptilian species, except lion, coyote and feathertail glider for which the hearts were not available.

### Sample preparation and protein analysis

All of the methods for sample preparation, gel electrophoresis for examination of MHC and MLC isoforms, dot blots, extraction of myosin from single muscle fibers for identification of MLC isoforms, and mass spectrometry were identical to those described previously (Reiser et al., 2009). Two separating gel formats, with 7% acrylamide, were used to separate MHC isoforms in this study, as in a recent study (Reiser et al., 2009). These formats were used in previous studies to optimally separate MHC isoforms in skeletal muscles (‘Format A’) (Bicer and Reiser, 2004) or in cardiac atria and ventricles (‘Format B’) (Reiser and Kline, 1998) and differ only with respect to glycerol content – 30% and 5% (v/v) in Format A and Format B, respectively. The same stacking gel (with 5% glycerol) was used with both separating gel formats. All other gel parameters and the running conditions are as described earlier: Format A (Bicer and Reiser, 2004); Format B (Reiser and Kline, 1998). Format B gels were used for samples of skeletal and cardiac muscles of those species in which the predominant MHC isoform in jaw-closing muscles co-migrated with cardiac MHC-α or with fast-type MHC from limb muscles on Format A gels. Proteins used for mass spectrometry were excised from one-dimensional slab gels (for most species) or two-dimensional (2-D) gels (cat and dog). Isoelectric focusing for 2-D gels was performed in a PROTEAN® IEF Cell (Bio-Rad, Inc, Hercules, CA, USA) using the Bio-Rad ReadyPrep™ 2-D Starter Kit, according to the manufacturer’s instructions. The pH gradient of the 11 cm Bio-Rad ReadyStrip™ IPG strips was 4.0–7.0. All of the gels from which protein bands or spots were excised were stained as described earlier (Reiser and Bicer, 2006).

### RNA analysis

Total RNA was extracted from masseter, temporalis and right atrium samples using the TRIzol method (Invitrogen, Carlsbad, CA, USA). cDNAs from these samples were produced by reverse transcription with random primers. The PCR primer pair designed to target mRNA sequences of MLC1E/A was: 5′-ACCCAAGCCTGAAGAGATG-3′ and 5′-CTCATCTTCTCTCCCAG-3′. PCR was carried out by using the primer pair and cDNA templates generated from the muscle tissues. The criterion for choosing these primers was described previously (Reiser et al., 2009). The PCR products were purified using a Qiaquick PCR purification kit (Qiagen, Valencia, CA, USA). The purified PCR amplicons were cloned into PCR 2.1-TOPO vector by TOPO TA cloning (Invitrogen). The cloned cDNAs were sequenced using an automatic sequencer (Plant-Microbe Genomic Facility at Ohio State University). The sequence data were aligned with the known sequences of rat MLC1E/A (NM_00119495.1), using a two-sequence blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The new sequences for raccoon, dog and cat MLC1E/A mRNA were submitted to GenBank and the following accession numbers were assigned: GU143840, GU143841, and GU143842, respectively.

## RESULTS

### Identification of masticatory myosin heavy chain

The expression of MHC-M in jaw-closing muscles of all nineteen species in this study was either tested or was verified, the latter based upon previous reports by others, with dot blots, using an antibody (2F4) that is specific for MHC-M (Kang et al., 1994). A dot blot for MHC-M for cat, coyote, macaque and bat, is shown in Fig. 1. Dot blots for additional species are shown in supplementary material Fig. S1. These blots identified the presence of MHC-M in every jaw-closing muscle tested in all nineteen species and failed to detect MHC-M in any cardiac or limb muscle sample. The dot blot results, as well as the results from all of the other approaches in this study were identical for the masseter muscle and the temporalis muscle of the same species, whenever both were studied.

The electrophoretic mobility of the MHC isoform(s) in jaw-closing muscles was compared with that of MHC isoforms in fast and slow limb muscles and in cardiac atria and ventricles, using two gel formats which optimally separate MHC isoforms in either mammalian skeletal muscle (Format A) or in mammalian cardiac muscle (Format B; see Materials and methods). A prominent MHC isoform, that had a unique electrophoretic mobility on Format A gels, compared with that of cardiac MHC isoforms and of fast and slow limb muscle MHC isoforms, was observed in jaw-closing muscles of domestic cat, macaque, Virginia opossum, tiger quoll and sugar glider (illustrated for domestic cat and dog in Fig. 2; Format A gels for all other species are shown in supplementary

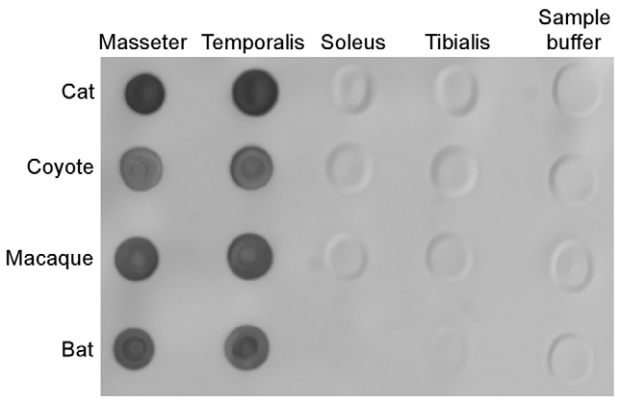



Fig. 1. Dot blot of homogenates of the masseter, temporalis, soleus and tibialis cranialis from domestic cat, coyote, long-tailed macaque and big brown bat with antibody 2F4 that is specific for masticatory myosin heavy chain. Sample buffer, without protein, was blotted onto the membrane as a control for the color of the sample buffer because of the presence of Bromophenol Blue. The antibody recognized the jaw-closing, but not limb, muscles in each species.

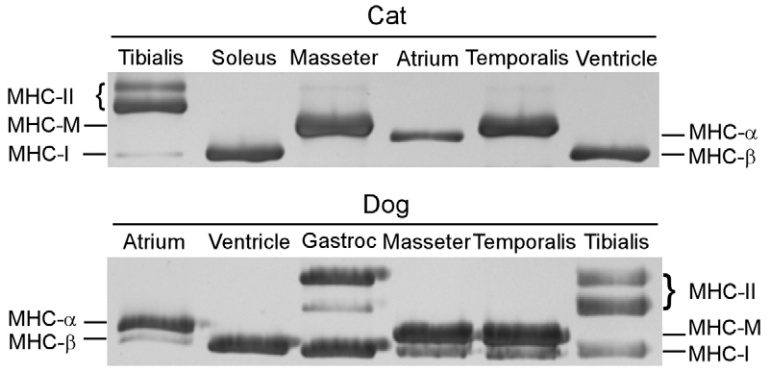



Fig. 2. Myosin heavy chain (MHC) region of two gels (Format A gels with 30% glycerol – see Materials and methods) loaded with homogenates of domestic cat and domestic dog fast tibialis cranialis, slow soleus (cat), masseter, cardiac atrium, temporalis, cardiac ventricle and the predominantly slow, deep portion of the gastrocnemius (dog). Other abbreviations: MHC-II, fast-type MHC isoforms; MHC-I, slow-type MHC; MHC-M, masticatory MHC; MHC-α, predominant MHC isoform in the atrium; MHC-β, predominant MHC isoform in the ventricle. Note that MHC-M and MHC-α from cats have subtly different electrophoretic mobilities and that the MHC-M band is observed only in jaw-closing muscles in cats on this gel. By contrast, a predominant MHC band with the same electrophoretic mobility is observed in dog atrium and jaw-closing muscles, using the same gel format. However, dog MHC-M and MHC-α can be separated using another gel format (Format B with 5% glycerol–see Materials and methods), as shown in Fig. 3.

material Fig. S2). The prominent band in jaw-closing muscles of domestic dog, raccoon, bat and skunk co-migrated only with the predominant band in the atria of each species and, in the gray short-tailed opossum the predominant jaw-closing band co-migrated with one of the fast-type bands in limb muscles on Format A gels. Therefore, samples from all of these species were run on Format B gels and the predominant jaw-closing band migrated differently from the bands with which they co-migrated on Format A gels (illustrated for dog in Fig. 3 and for raccoon, skunk, bat and gray short-tailed opossum in supplementary material Fig. S3). Turtle (painted, softshell, map, snapping and red-eared slider) and alligator samples were run on Format B gels because this format yielded much better separation of all of the observed bands, compared with Format A gels. The predominant jaw-closing band in all of the turtle species and alligator had a unique electrophoretic migration relative to the MHC isoforms in cardiac atria and ventricle and in fast and slow limb muscles. Cardiac samples were not available from the lion, coyote and feathertail glider, so it cannot be stated that the predominant jaw-closing isoform has unique electrophoretic mobility, compared with cardiac MHC isoforms, in these species. In addition, the predominant MHC isoform in lion masseter and temporalis had an electrophoretic mobility that was distinct from slow MHC-I and one fast-type MHC isoform in leg muscle, but had the same mobility as another fast-type isoform. The dot blot results from all of the species, corroborated by the electrophoretic mobility patterns in the majority of species, indicate that every species selected for this study does, in fact, express MHC-M as the predominant or exclusive MHC isoform in jaw-closing muscles. This extends the range of species known to express masticatory myosin, with the addition of raccoon, lion, coyote, big brown bat, tiger quoll, feathertail glider, sugar glider, American alligator, common snapping turtle, spiny softshell turtle, painted turtle, common map turtle and red-eared slider to those species already reported by others.

### Identification of myosin light chain 1 isoforms

Myosin was extracted from skinned fast and slow fibers from limb muscles, skinned fibers from jaw-closing muscles, and skinned atrial and ventricular strips, of all species except those for which cardiac samples were not available (lion, coyote and feathertail glider) or in which slow fibers could not be identified (gray short-tailed opossum). The extracted myosin was used as a source of MLC standards to assist in the identification of MLC isoforms in jaw-closing muscles, which was based upon electrophoretic mobility and known stoichiometry of MLC proteins in skeletal and cardiac muscles (illustrated for cat in Fig. 4A and for red-eared slider in Fig. 5). The predominant or exclusive MLC1 isoform in jaw closing muscles of domestic cat, domestic dog, raccoon, skunk, macaque, bat, gray short-tailed opossum, Virginia opossum, tiger quoll and sugar glider co-migrated with MLC1 in the atrium, and not with either fast-type or slow-type MLC1 in limb muscles (illustrated for cat in Fig. 4B). Reptiles appear to express the same isoform(s) of MLC1 and of MLC2 throughout the entire heart (Fig. 6 and Discussion). Therefore, the MLC1 in jaw-closing muscles of reptiles should be referred to simply as embryonic, not atrial, MLC1. Consistent with this, we observed only a single MLC1 protein band throughout the heart (atria and ventricle) of the alligator (Fig. 6) and each of the five turtle species (Fig. 7), and the MLC1 isoform in jaw-closing muscles of all of the reptile species studied had an electrophoretic mobility that was different from the cardiac MLC1 isoform and different from fast-type MLC1 in limb muscles.

Mass spectrometry (MS) was used to identify the isoform of MLC1 in the jaw-closing muscles of all 13 mammalian species, the alligator and all five turtle species. Gel bands (from 1-D gels) or gels spots (from 2-D gels) were excised for analysis by mass spectrometry. A 2-D gel which was used to isolate MLC1 from cat temporalis is shown in Fig. 8. The protein with the greatest match in most species was the embryonic/atrial isoform of MLC1 (Table 2). The only exceptions were painted turtle and softshell turtle in which

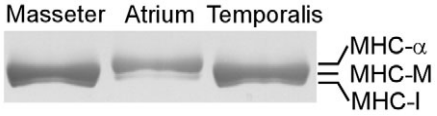



Fig. 3. Myosin heavy chain (MHC) region of a Format B gel (see Materials and methods) loaded with homogenates of domestic dog masseter, cardiac atrium and temporalis. Other abbreviations: MHC-α, predominant MHC isoform in cardiac atria; MHC-M, masticatory MHC; MHC-I, slow-type MHC. Note the different mobilities of dog MHC-α and MHC-M on this gel format, in contrast to their very similar mobility on Format A gels (Fig. 2).

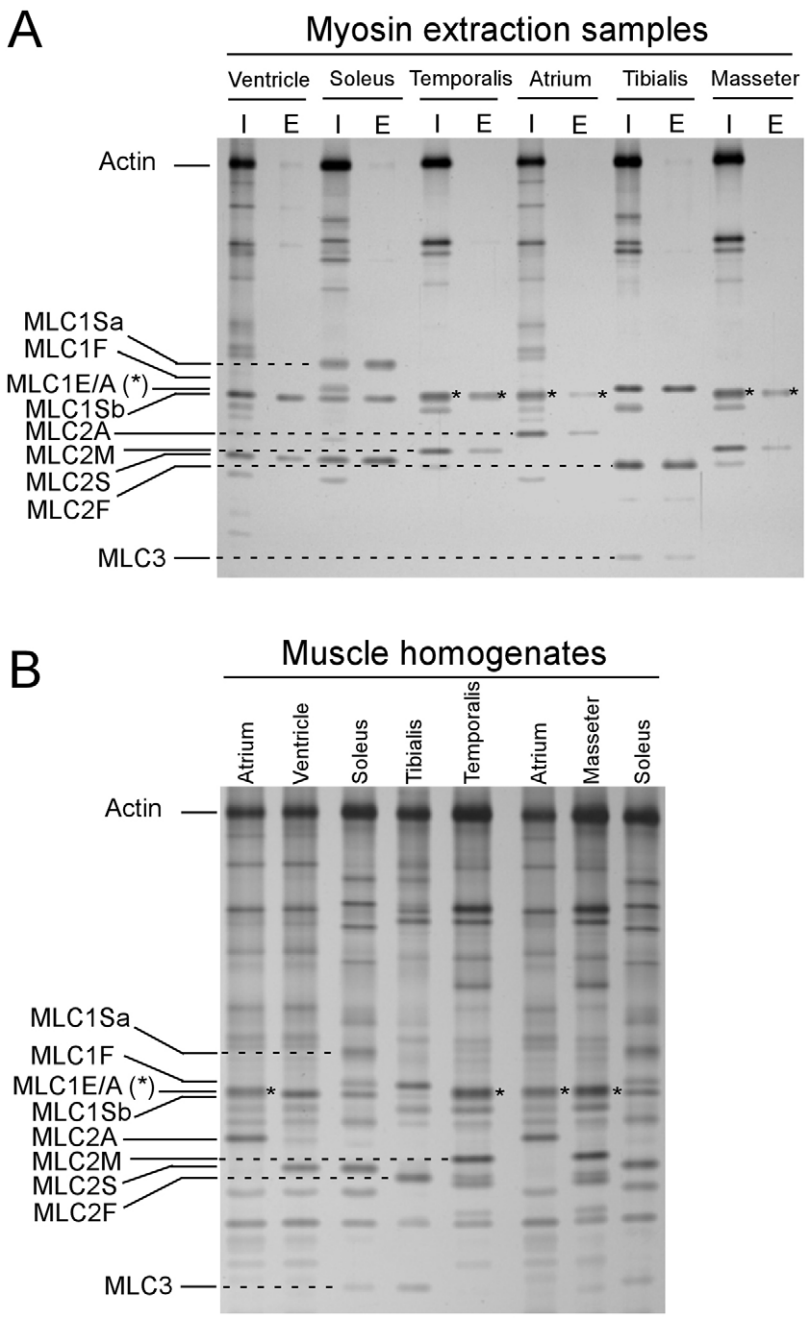



Fig. 4. The low molecular mass region of SDS gels onto which cat samples that were used for myosin extractions (A) or non-extracted muscle homogenates (B) were loaded. Myosin was extracted from thin, skinned strips of cardiac ventricle and atrium and skinned single fibers from soleus, temporalis, tibialis cranialis and masseter muscles. One-half of each strip or single fiber was intact (not extracted) and loaded onto the 'I' lane. The extracted myosin from the other half of the strip or fiber was loaded onto the adjacent 'E' lane. The identified myosin light chain (MLC) isoforms were two slow-type MLC1 isoforms (MLC1Sa and MLC1Sb) (Sarkar et al., 1971; Weeds, 1976), fast type MLC1 (MLC1F), embryonic/atrial MLC1 (MLC1E/A – bands denoted with asterisks), masticatory MLC2 (MLC2M), slow type MLC2 (MLC2S), fast-type MLC2 (MLC2F) and MLC3. The predominant MLC1 isoform in temporalis, atrium and masseter, with identical mobility in these three samples, is indicated with an asterisk at the right-hand edge of the protein bands in A and in B.

MS analysis yielded slightly higher MOWSE scores for triosephosphate isomerase than MLC1E/A in the same sample runs. It is possible that the excised bands for these two species contained triosephosphate isomerase, or a fragment thereof, along with MLC1E/A, because none of the trypsin-generated peptides were shared as the basis for the identification of these two proteins. Nevertheless, MLC1E/A was identified as the predominant MLC1 isoform in jaw-closing muscles of these species, as well.

PCR was used to complement the identification of MLC1E/A in three species – domestic cat, domestic dog and raccoon. Results of PCR amplification that targeted MLC1E/A are shown in Fig. 9. Raccoon right atrium was used as a positive control. Amplicons at the expected size (210 bp, predicted from rat MLC1E/A) were produced by PCR amplification of mRNAs extracted from the masseter and left atrium of the raccoon and the masseter and temporalis of cat and dog. No PCR product was generated using the fast tibialis cranialis (raccoon, cat, dog), slow soleus (raccoon, cat), or deep gastrocnemius (dog) mRNA as a template. The results of sequence alignment are also shown in Fig. 9. The raccoon, cat and dog sequences obtained from the MLC1E/A amplicons were 89–92% homologous to the rat MLC1E/A mRNA sequence. These results fully corroborate the protein analyses, indicating that MLC1E/A is the MLC1 isoform associated with MHC-M in these three species.

### Identification of myosin light chain 2 isoforms

The MLC2 isoform in jaw-closing muscles of the 13 mammalian species had an electrophoretic mobility that was distinct from the mobility of fast-type and slow-type MLC2 in limb muscles and of MLC2 in cardiac samples (illustrated for cat in Fig. 4), the exceptions for the later being lion, coyote and feathertail glider, for which cardiac samples were not available.

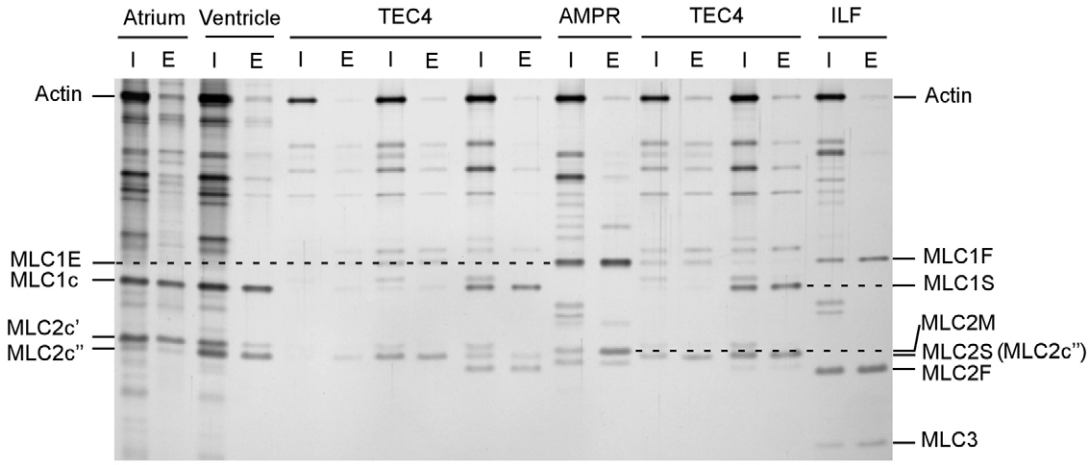



Fig. 5. The low molecular mass region of an SDS gel onto which red-eared slider samples that were used for myosin extractions were loaded. Myosin was extracted from thin, skinned strips of cardiac ventricle and atrium and from skinned single fibers from the fourth head of the testo-cervicis (TEC4), pars profunda of the external adductor (AMPR) and the iliofibularis (ILF) muscles. One-half of each strip or single fiber was intact (not extracted) and loaded into the 'I' lane. The extracted myosin from the other half of the strip or fiber was loaded into the adjacent 'E' lane. The myosin light chain (MLC) isoforms identified were cardiac MLC1 (MLC1c), two cardiac MLC2 isoforms (MLC2c′ and MLC2c″), fast type MLC1 (MLC1F), slow type (MLC1S), embryonic MLC1 (MLC1E), masticatory MLC2 (MLC2M), slow type MLC2 (MLC2S, co-migrates with MLC2c″), fast-type MLC2 (MLC2F) and MLC3.

Several observations were consistent for each reptile species examined. Examination of the myosin extracted from the atria and ventricle of all of the reptiles consistently suggested that there are two isoforms of MLC2 throughout the entire heart, designated as MLC2c′ and faster migrating MLC2c″ (illustrated for red-eared slider in Fig. 5, and for alligator and all turtle species in Figs 6 and 7, respectively). Generally, MLC2c′ predominated in the atria and MLC2c″ predominated in the ventricle. MLC2 in jaw-closing muscles of all reptiles migrated differently from fast-type MLC2 in limb muscle and from MLC2c′ and MLC2c″.

The MLC2 isoform of alligator jaw-closing muscles migrated slightly slower than MLC2c′ (Fig. 6). Therefore, the mobility of alligator jaw-closing MLC2 was distinct from all other MLC2 isoforms (fast, slow and cardiac), as in the examined mammalian species.

The myosin extraction protocol, when applied to turtle slow limb fibers, yielded multiple protein bands in the MLC2 region on gels. The identity of slow-type MLC2 in turtle muscles is, therefore, not firm. For example, there appeared to be two slow-type MLC2 isoforms in some fibers from the fourth head of the testo-cervicis in the red-eared slider, one isoform of which co-migrated with MLC2c″ in the heart (Fig. 5). Nevertheless, MLC2 in red-eared slider jaw-closing muscles migrated differently from all of the putative slow-type MLC2 isoforms in limb muscle (Fig. 5). Therefore, MLC2 in red-eared slider jaw-closing muscles appears to be distinct from all other MLC2 isoforms in this species. Owing to the complexity of slow-type MLC2 isoform expression in the other turtle species, clear statements cannot be made concerning whether MLC2 in their jaw-closing muscles is distinct.

## DISCUSSION

The results of this study reveal that masticatory myosin is more broadly expressed across several vertebrate orders than previously reported. MHC-M is exclusively or predominantly expressed in each of these species. MHC-M had previously been reported to be expressed in 29 vertebrate species (Rowlerson et al., 1981; Rowlerson et al., 1983a; Rowlerson et al., 1983b; Sciote and Rowlerson, 1998; Sciote et al., 1995; Hoh, 2002; Hoh et al., 2006). The results increase the number of vertebrate species that are known to express masticatory myosin by 14, including six from Reptilia and eight additional eutherian and marsupial mammalian species.

The results also revealed that the MLC1 isoform that is expressed in association with MHC-M, in every species examined, is MLC1E/A. This isoform in reptiles should be referred to simply as the embryonic, not the atrial, isoform of MLC1 because a single MLC1 isoform, MLC1c, was detected in the atria and ventricle of all six species of Reptilia studied, consistent with an earlier report (Oh-Ishi and Hirabayashi, 1989). We recently reported (Reiser et al., 2009) that MLC1E/A is expressed in association with MHC-M in jaw-closing muscles of some members of Sciuridae. This was

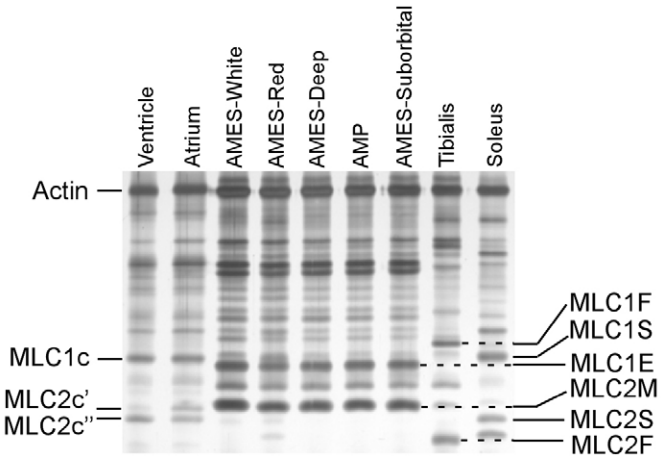



Fig. 6. The low molecular mass region of an SDS gel loaded with alligator muscle homogenates. Each lane was loaded with a homogenate of ventricle or atrium, or with the white portion, red portion, deep portion or a suborbital region of the adductor mandibulae externus (AMES), adductor mandibulae posterior (AMP), tibialis cranialis or soleus muscles. The myosin light chain (MLC) isoforms identified were cardiac MLC1 (MLC1c), two cardiac MLC2 isoforms (MLC2c′ and MLC2c″), fast type MLC1 (MLC1F), slow type MLC1 (MLC1S), embryonic MLC1 (MLC1E), masticatory MLC2 (MLC2M), slow type MLC2 (MLC2S, co-migrates with MLC2c″) and fast-type MLC2 (MLC2F).

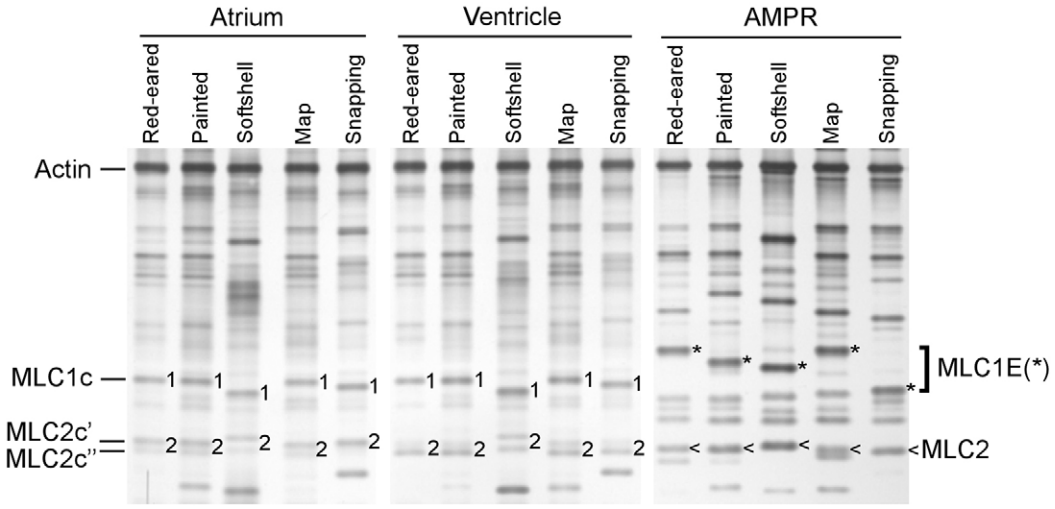



Fig. 7. The low molecular mass region of an SDS gel loaded with turtle muscle homogenates. Each lane was loaded with a homogenate of cardiac atrium, ventricle or pars profunda of the external adductor (AMPR) muscle from red-eared slider turtle, painted turtle, softshell turtle, map turtle, or snapping turtle. The myosin light chain (MLC) isoforms identified were cardiac MLC1 (MLC1c), two cardiac MLC2 isoforms (MLC2c′ and MLC2c″) and embryonic MLC1 (MLC1E – bands denoted with asterisks) and MLC2 in the AMPR.

the first report of MLC1E/A being expressed with MHC-M. It was not known at that time whether this was a unique pattern among Sciuridae or was a more universal pattern. To address this we subsequently sampled several species in which MHC-M expression had previously been reported, plus 14 additional species. We conclude that it is unlikely that a unique masticatory isoform of MLC1 exists in any species and that it is likely that all species that express MHC-M also express MLC1E/A in the same muscles.

The existence of a unique masticatory isoform of MLC1 was initially proposed, based upon the observation that the electrophoretic mobility of MLC1 in cat temporalis muscle was different from that of fast-type and slow-type MLC1 in cat limb muscles (Rowlerson et al., 1981). However, the predominant MLC1 isoform in cat temporalis muscle was not compared to MLC1 in cat atria by Rowlerson and co-workers. A unique masticatory isoform of MLC2 (i.e. MLC2-M) was also reported (Rowlerson et al., 1981) and this protein, along with MHC-M, has been cloned and the deduced sequence reported (Qin et al., 1994; Qin et al., 2002). No direct evidence of a unique masticatory isoform of MLC1, corresponding to MLC2-M and MHC-M, has ever been reported.

MLC1E/A is expressed in adult human masseter (e.g. Rotter et al., 1991; Soussi-Yanicostas et al., 1990; Soussi-Yanicostas and Butler-Browne, 1991; Stål et al., 1994; Bontemps et al., 2002), along with primarily the slow-type MLC isoforms and MLC1F that are typically expressed in adult limb muscles, but MHC-M is not expressed in human masseter (Stedman et al., 2004). Therefore, while it appears that MLC1E/A is the MLC1 isoform that is always expressed with MHC-M, the converse (i.e. MHC-M always being expressed wherever MLC1E/A is expressed) is clearly not true. Adult human masseter expresses predominantly the slow isoform of MHC, along with small amounts of developmental (embryonic and neonatal), alpha cardiac, and fast-type (IIA and IID/X) MHC isoforms (Soussi-Yanicostas et al., 1990; Bredman et al., 1991; Pedrosa-Domellöf et al., 1992; Sciote et al., 1994; Stål, 1994; Monemi et al., 1996; Korfage et al., 2000; Yu et al., 2002; Gedrange et al., 2005; Rowlerson et al., 2005; Harzer et al., 2007). Expression patterns of MHC isoforms in mammalian jaw-closing muscles are, in general, more complex than in limb muscles of the same species (reviewed by Hoh, 2002; Sciote et al., 2003).

It is interesting to consider what has led to the coordinated expression of MLC1E/A, MLC2M and MHC-M in jaw-closing muscles of many vertebrates, as well as mechanisms that regulate myosin subunit gene expression in these muscles, given that a multitude of MHC and MLC genes are present in all species. MHC-M appears to be a distinct subclass of vertebrate sarcomeric MHC, as proposed by Qin and co-workers (Qin et al., 2002) who reported about only 70% homology between MHC-M and fast-type and slow-type MHC isoforms, whereas other vertebrate MHC isoforms share much greater homology. The designation of MHC-M as a distinct subclass is supported by chromosomal localization of the genes

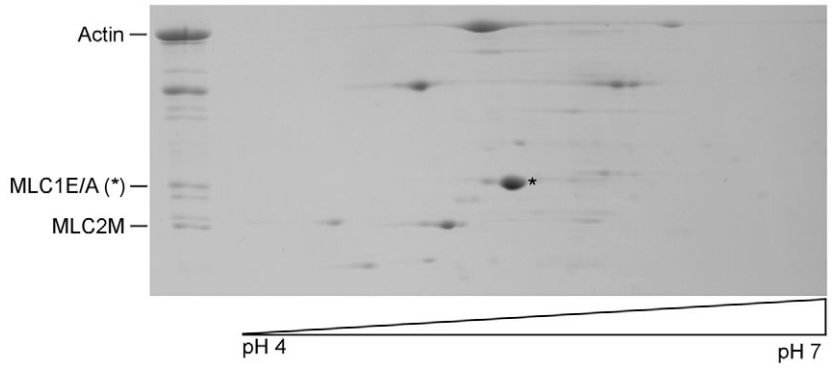



Fig. 8. Two-dimensional gel (pH gradient from 4.0 to 7.0 in the first dimension) loaded with a homogenate of cat temporalis muscle. The same sample was loaded in the reference lane on the left-hand edge of the gel. The embryonic/atrial myosin light chain (MLC1E/A) spot (indicated with an asterisk) on this gel was excised and analyzed by mass spectrometry. Other abbreviation: MLC2M, masticatory myosin light chain 2.

Table 2. Identification of myosin light chain 1 by mass spectrometry

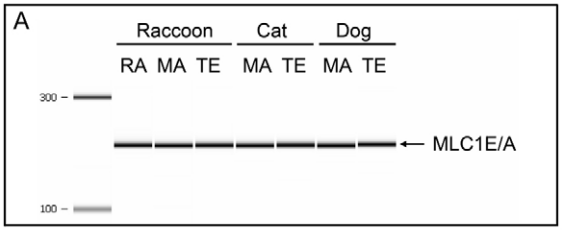
| Species | Protein matched | Peptides matched | PBMS* |
|---|---|---|---|
| Domestic cat | MYL4†, human | 12 | 840 |
| Lion | MYL4, dog | 16 | 1097 |
| Domestic dog | MYL4, dog | 18 | 1273 |
| Coyote | MYL4, dog | 17 | 1241 |
| Striped skunk | MYL4, dog | 14 | 1137 |
| Northern raccoon | MYL4, dog | 15 | 1030 |
| Long-tailed macaque | MYL4, rhesus monkey | 18 | 1222 |
| Big brown bat | MYL4, dog | 15 | 1137 |
| Gray short-tailed opossum | MYL4, rat | 10 | 736 |
| Virginia opossum | MYL4, rat | 10 | 744 |
| Tiger quoll | MYL4, rat | 10 | 699 |
| Feathertail glider | MYL4, rat | 8 | 520 |
| Sugar glider | MYL4, Human | 9 | 585 |
| American alligator | MYL4, rat | 8 | 578 |
| Common snapping turtle | MYL4, zebrafish | 5 | 389 |
| Spiny softshell turtle | MYL4, zebrafish | 5 | 414 |
| Painted turtle | MYL4, zebrafish | 6 | 436 |
| Common map turtle | MYL4, zebrafish | 5 | 412 |
| Red-eared slider turtle | MYL4, human | 6 | 354 |

*Probability-based MOWSE score.
†Embryonic/atrial isoform of myosin light chain 1.

encoding MHC isoforms. Whereas fast-type MHC genes are located on human chromosome 17 (Weiss et al., 1999) and cardiac alpha and beta MHC genes are located on human chromosome 14 (Saez et al., 1987), *MYH16*, which encodes MHC-M, is located on human chromosome 7 [Yu et al. (Yu et al., 1996) cited in Qin et al. (Qin et al., 2002)].

Masticatory myosin consists of a unique heavy chain isoform and a unique MLC2 isoform (Rowlerson et al., 1981; Qin et al., 1994; Qin et al., 2002; Hoh et al., 2007). The MLC1 isoform of masticatory myosin is, however, not unique as it is the same isoform that is expressed in skeletal and cardiac muscles during embryonic development, as well as in atria of adult mammals (Reiser et al., 2009) (present study). MHC-M is believed to be an evolutionarily ancient protein, as discussed by Qin and co-workers (Qin et al., 2002). It is interesting to consider the association of MLC1E/A with MLC-M, as this light chain isoform is expressed in mammalian skeletal and heart muscles at embryonic stages (Whalen et al., 1978; Whalen and Sell, 1980), some slow fibers in adult dog extraocular muscles (Bicer and Reiser, 2004; Bicer and Reiser, 2009), some slow fibers in adult dog thyroarytenoid muscle (Bergrin et al., 2006), as well as human masseter which does not express MHC-M (discussed above). Given the broad phylogenetic distribution of MLC2-M expression, including members of Carnivora (Rowleson et al., 1981; Qin et al., 1994) (this study), alligator (this study), as well as some rodent species (Reiser et al., 2009), it appears that MLC2M is also a protein that arose at about the same time as MHC-M. The MLC1E/A isoform also appears to be an early isoform, given its broad phylogenetic expression. It appears that a change in its expression occurred when the mammalian lineage arose as it is expressed in the atria of all studied mammalian species but not in the atria of birds and amphibians (Grandier-Vazeille et al., 1983), or reptiles (present study).

The objective of this study was to determine whether MLC1E/A is consistently expressed with MHC-M in vertebrate jaw-closing muscles. Observations were also made on the electrophoretic mobility of MLC2 in the same jaw-closing muscles. MLC2M is already known to be expressed in cat temporalis muscle (Rowlerson et al., 1981; Shelton et al., 1985; Qin et al., 1994), as well as in jaw-closing muscles of some members of Sciuridae (Reiser et al., 2009). It appears that MLC2M is expressed in the jaw-closing muscles of all of the mammalian species examined in this study, as well as the American alligator and red-eared slider, given the distinct mobility of MLC2 in these species. It seems probable that MLC2M is also expressed in the jaw-closing muscles of the other turtle species examined, given its distinct electrophoretic mobility with respect to fast-type and cardiac MLC2 isoforms. However, a limitation of this study is that the myosin extraction protocol yielded multiple proteins that migrated in the MLC2 region on gels and it is not clear if MLC2 in the jaw-closing muscles of four of the turtle species is a unique protein. It is possible that multiple slow-type MLC2 isoforms are expressed in turtle limb muscle and/or the extraction protocol is not as selective in turtles as it is in mammals. Therefore,




| B | Sequence Homology | | | | | |
|---|---|---|---|---|---|---|
| | Raccoon (%) | | Cat (%) | | Dog (%) | |
| MLC1 E/A | MA | TE | MA | TE | MA | TE |
| (Rat; NM_00119495.1) | 92 | 92 | 92 | 92 | 89 | 89 |

Fig. 9. Identification of embryonic/atrial myosin light chain 1 (MLC1E/A) mRNA sequences in raccoon, domestic cat and domestic dog. (A) Electrophoresis results of PCR-amplified MLC1E/A mRNA sequences generated from right atrium (RA), masseter (MA) and temporalis (TE) of raccoon, cat and dog. Amplicons at the expected size (210 bp) are indicated by an arrow. (B) Sequence homologies of the putative raccoon, cat and dog MLC1E/A mRNA sequences with the published sequence in rat.

except in the red-eared slider, it cannot be determined, from the present results, whether MLC2M or slow-type MLC2S is expressed with MHC-M in turtles.

Much progress has been made in understanding the role of MLCs in regulating contractile properties of striated muscle (for a review, see Timson, 2003). However, the functional significance of the association of MLC1E/A with MHC-M and MLC2M is unclear. As discussed previously (Reiser et al., 2009), the affinity of MLC1E/A for actin is lower than that of slow-type MLC1 (Morano and Hasse, 1997) and this could allow for more rapid cross-bridge cycling during activation. This, in turn, could augment power output of fibers expressing MLC1E/A, compared to a hypothetical combination of MLC1S with MHC-M. It is possible that the association of MLC1F with MHC-M would compromise force production or power generation, or other contractile properties, that might otherwise be optimized by the association of MLC1E/A with MHC-M. It is also possible that the association of MLC1E/A with MHC-M and/or MLC2M is not driven by a mechanical advantage but rather is governed by a developmental expression program that is, at least partially, retained in adult jaw-closing muscles. Additional investigations will be required to fully understand the functional significance of the apparent consistent association of MLC1E/A with MHC-M across a broad range of species.

## LIST OF ABBREVIATIONS

| | |
|---|---|
| MHC | myosin heavy chain |
| MHC-I | slow-type myosin heavy chain |
| MHC-M | masticatory myosin heavy chain |
| MLC1 | myosin light chain 1 |
| MLC1c | reptilian cardiac myosin light chain 1 |
| MLC1E/A | embryonic/atrial myosin light chain 1 |
| MLC1F | fast-type myosin light chain 1 |
| MLC1S | slow-type myosin light chain 1 |
| MLC2 | myosin light chain 2 |
| MLC2c′, MLC2c″ | reptilian cardiac myosin light chain 2 isoforms |
| MLC2F | fast-type myosin light chain 2 |
| MLC2M | masticatory myosin light chain 2 |
| MLC2S | slow-type myosin light chain 2 |
| *MYH16* | masticatory myosin heavy chain gene |

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