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# Masticatory ('superfast') myosin heavy chain and embryonic/atrial myosin light chain 1 in rodent jaw-closing muscles

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## SUMMARY

**Masticatory myosin is widely expressed among several vertebrate classes. Generally, the expression of masticatory myosin has been associated with high bite force for a carnivorous feeding style (including capturing/restraining live prey), breaking down tough plant material and defensive biting in different species. Masticatory myosin expression in the largest mammalian order, Rodentia, has not been reported. Several members of Rodentia consume large numbers of tree nuts that are encased in very hard shells, presumably requiring large forces to access the nutmeat. We, therefore, tested whether some rodent species express masticatory myosin in jaw-closing muscles. Myosin isoform expression in six Sciuridae species was examined, using protein gel electrophoresis, immunoblotting, mass spectrometry and RNA analysis. The results indicate that masticatory myosin is expressed in some Sciuridae species but not in other closely related species with similar diets but having different nut-opening strategies. We also discovered that the myosin light chain 1 isoform associated with masticatory myosin heavy chain, in the same four Sciuridae species, is the embryonic/atrial isoform. We conclude that rodent speciation did not completely eliminate masticatory myosin and that its persistent expression in some rodent species might be related to not only diet but also to feeding style.**

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/212/16/2511/DC1

Key words: masticatory myosin, myosin heavy chain, myosin light chain, masseter, temporalis, rodents.

## INTRODUCTION

Myosin in striated muscles consists of two high molecular weight heavy chains, MHC, and two pairs of light chains, MLC. There is extensive heterogeneity in the isoform expression of MHC and of MLC among vertebrate species, in general, and among individual muscle cells within a given organism. Substantial evidence, obtained through a variety of experimental approaches, clearly demonstrates that MHC and MLC isoforms have important roles in determining physiological properties of muscle. Craniofacial muscles (jaw-closing, extraocular and laryngeal) express MHC and MLC isoforms that are present in limb muscles that drive locomotion, plus other MHC and MLC isoforms that are not typically expressed in limb muscles. Masticatory myosin, initially referred to as 'superfast myosin' (Rowlerson et al., 1983), is expressed in jaw-closing muscles of multiple species in several vertebrate classes, including mammals (orders Carnivora, Primates, Chiroptera, Didelphimorphia, Dasyuromorphia, Diprotodontia), reptiles (Crocodylia, Chelonian) and fish (Pleurotemata) (reviewed by Hoh, 2002; Hoh et al., 2006). More recent reports (reviewed by Yamaguchi, 2007; Reiser and Bicer, 2007; Toniolo et al., 2008) indicate that this myosin imparts high force-generating ability, not high contractile speed, to fibers in which it is expressed. Therefore, the term 'masticatory myosin' is used in this report, consistent with the suggestion by Hoh (Hoh, 2002) and Qin et al. (Qin et al., 2002). Given the feeding styles of several of the species in which masticatory myosin is expressed (e.g. sharks, crocodiles and members of the cat family that hunt large prey), it has been readily accepted that rodents, with very different feeding styles, do not express masticatory myosin. This general acceptance has accrued because reports during the past two decades have accurately demonstrated that the myosin expressed in jaw-closing muscles of the rodent species, which have been studied thus far (mouse, rat, guinea pig), consists predominantly of those isoforms that are typically expressed in neonatal and adult fast and slow limb muscles, as well as small amounts of MHC-$\alpha$ in adult mouse masseter (e.g. d'Albis et al., 1986; Eason et al., 2000; Widmer et al., 2002) (also see Discussion). No evidence has shown that any rodent species expresses masticatory myosin in any muscle. The latter is especially noteworthy because rodents comprise about 40% of all extant mammalian species. The apparent absence of masticatory myosin in the entire rodent order has interesting evolutionary and phylogenetic implications and raises several interesting questions, such as what has driven the apparent complete repression of masticatory myosin expression in this order and, if some rodents species do, in fact, express masticatory myosin, is the same true for other, closely related species.

We examined patterns of MHC and MLC isoform expression in jaw-closing muscles of six rodent species, all being members of the family Sciuridae, using protein gel electrophoresis, mass spectrometry, immunoblotting and analysis of mRNA sequences. The results definitively demonstrate that the masticatory isoform of MHC (MHC-M) and of MLC2 (MLC2M), as well as the embryonic/atrial isoform of MLC1 (MLC1E/A), are the predominant or exclusive isoforms of myosin in the jaw-closing muscles in eastern gray squirrels, eastern fox squirrels, woodchucks and eastern chipmunks, and that MHC-M, MLC2M and MLC1E/A are not expressed in jaw-closing muscles of southern flying squirrels or red squirrels. These results show that

masticatory myosin is expressed in the order Rodentia and is, therefore, more pervasive among vertebrates than previously realized.

## MATERIALS AND METHODS

### Samples

Samples of masseter, temporalis, tibialis cranialis, soleus, left atrium and left ventricle were isolated from captured eastern gray squirrels (*Sciurus carolinensis* Gmelin, *N*=4), eastern chipmunks (*Tamias striatus* Linnaeus, *N*=3), southern flying squirrels (*Glaucomys volans* Linnaeus, *N*=3), red squirrel (*Tamiasciurus hudsonicus* Erxleben, *N*=3), eastern fox squirrel (*Sciurus niger* Linnaeus, *N*=2), woodchuck (*Marmota monax* Linnaeus, *N*=1) and Virginia opossum (*Didelphis virginiana* Kerr, *N*=1). The animals were captured by a local animal control company and were euthanized at the company facility by exposure to carbon dioxide, according to company policies and in accordance with the recommendations of the American Veterinary Medical Association Panel on Euthanasia. All captured animals appeared to be adults, based upon body mass, and were of either gender. Samples were obtained immediately after euthanasia and transported on crushed ice or dry ice (the latter for samples for mRNA sequence analysis) to the laboratory for subsequent preparation. Samples were also obtained from one normal adult male domestic dog (hound), which served as positive controls for masticatory myosin on immunoblots. The care and use of the dog, including euthanasia, were in accordance with a protocol approved by the Institutional Animal Care and Use Committee of The Ohio State University. The dog was deeply anesthetized prior to cardiectomy. Samples for protein analysis were obtained immediately following removal of the heart and placed in cold relaxing solution (Bergrin et al., 2006) and transported on ice to the laboratory within one hour following euthanasia. Bundles of fibers, as well as thin (~1 mm thick) strips of the left atrium and left ventricle of each species were immediately dissected in the same solution and stored in glycerinating solution (Bergrin et al., 2006) at –20°C. The glycerinated samples served as a source of skinned fibers. Portions of some of the muscle samples were immediately frozen at –40°C until they were prepared for gel electrophoresis. Myosin was extracted from single skinned fibers and cardiac strips, as described in Bicer and Reiser (Bicer and Reiser, 2004). The extracted myosin was run on an initial set of 12% acrylamide gels to identify MLC isoforms on subsequent gels that were loaded with the same homogenates that were used for MHC isoform analysis.

### Gel electrophoresis and immunoblotting

All electrophoretic procedures were as described in Bicer and Reiser (Bicer and Reiser, 2004) and in Reiser and Kline (Reiser and Kline, 1998), except the cross-linking ratio in the separating gels that were used to separate low molecular weight proteins for gray squirrel, fox squirrel, chipmunk and woodchuck samples was 50:1, which yielded slightly greater separation of some MLC isoforms. Scanning densitometry was used to determine the relative amount (percentage of total) of individual MHC isoforms in some of the samples, as described in Bicer and Reiser (Bicer and Reiser, 2004). Myosin was extracted from multiple single, skinned fibers from the slow-twitch soleus, fast-twitch tibialis cranialis and masseter muscles of each species included in this study and run on initial gels (see Figs S1–S6 in supplementary material) as standards to identify MLC isoforms in samples on subsequent gels, as in Bicer and Reiser (Bicer and Reiser, 2004). Monoclonal antibodies against MHC-M [2F4, characterized in Kang et al. (Kang et al., 1994)] and MHC-β/I [A4.951, characterized in Webster et al. (Webster et al., 1988)] were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, IA, USA. A monoclonal antibody [F88-12F8, characterized by Peuker et al. (Peuker et al., 1999)] against MHC-α was obtained from Alexis Biochemicals (AXXORA, LLC, San Diego, CA, USA). Western blots, using the anti-MHC-M antibody were run as described in Bergrin et al. (Bergrin et al., 2006). A dot immunoblot was used to test whether woodchuck masseter expressed anti-MHC-α, because in only this species a MHC isoform in the masseter co-migrated with the predominant MHC band in the left atrium when samples were run on a gel that separated cardiac MHC isoforms (Reiser and Kline, 1998). Three μl of the same samples as used for SDS-PAGE were applied to a nitrocellulose membrane and allowed to air dry. These samples contained bromophenol blue as a tracking dye. Therefore, a sample of gel buffer, without protein, was also applied to the membrane and served as a negative control for any signal due to bromophenol blue. An image of the dried membrane was captured before the membrane was soaked in blocking solution. All subsequent steps were as described for western blots in Bergrin et al. (Bergrin et al., 2006).

### Mass spectrometry

Mass spectrometry was used to assist in the identification of several proteins. Coomassie Blue-stained gel bands were excised and analyzed with liquid chromatography, coupled to tandem mass spectrometry, in the Campus Chemical Instrument Center at The Ohio State University, as described in Reiser and Bicer (Reiser and Bicer, 2006).

### RNA analysis

Total RNA was extracted from masseter, tibialis cranialis and left atrium samples using the conventional TRIzol method. cDNAs from these samples were produced by reverse transcription with random primers. The following PCR primer pairs were designed to target mRNA sequences of MLC1E/A, MLC2M and MHC-M: 5′-ACC-CAAGCCTGAAGAGATG/5′-CTCATCTTCTCTCCCAG (MLC-1E/A); 5′-CCTCATCCAATGTCTTCTCCAAC/5′ GAACTGGAA-CATCTGGTCCAC (MLC2M); 5′-CATGGGCATCTTCTC-CATCTTG/5′-CTCTTCTCCTTCTGGAGCTTG (MHC-M). These PCR primer pairs were designed from published mRNA sequences of rat MLC1E/A (accession number: NM_001109495), cat MLC2M (accession number: NM_001009874) and cat MHC-M (accession number: NM_001009221), respectively. The specific primer sequences were chosen by the criterion that these sequences were conserved in multiple species. The primers for MLC1E/A, for example, align perfectly with the mRNA sequences of MLC1E/A from rat, mouse, dog and human. PCR was carried out using these primer pairs and cDNA templates generated from the muscle tissues. The PCR products were analyzed by electrophoresis using a Bioanalyzer (Agilent, Santa Clara, CA, USA). The PCR amplicons were then cloned into PCR 2.1-TOPO vector by TOPO TA cloning (Invitrogen, Carlsbad, CA, USA). The cloned cDNAs were sequenced by an automatic sequencer (Plant–Microbe Genomics Facility at Ohio State University). Sequence data were aligned with the known sequences of rat MLC1E/A, cat MLC2M and cat MHC-M by the two-sequence blast (http://blast.ncbi.nlm.nih.gov/bl2seq/wblast2.cgi). The new sequences were submitted to GenBank and the following accession numbers were assigned: eastern gray squirrel MLC1E/A, FJ876275; MLC2M, FJ876277; and MHC-M, FJ876988; eastern chipmunk MLC1E/A, FJ876276; MLC2M, FJ876278; and MHC-M, FJ876279.

## RESULTS

### MHC and MLC isoforms in gray squirrels

The initial observation of the present study was the unique electrophoretic mobility of the predominant MHC isoform in samples of eastern gray squirrel masseter and temporalis muscles. This isoform had a different mobility, on separating gels containing 30% glycerol, compared with the mobility of MHC isoforms in tibialis cranialis (fast-twitch), soleus (slow-twitch), left atrium and left ventricle (Fig. 1A). This isoform was the predominant isoform in all regions of the masseter superficialis (superficial and deep layers), masseter profundus and temporalis (superficial and deep layers) in this species (not shown). The migration of this isoform was most similar to that of the slower migrating cardiac MHC isoform, presumed to be the alpha isoform of MHC (MHC-α). An immunoblot with an anti-MHC-α antibody revealed that the slower migrating cardiac MHC isoform (the predominant isoform in the gray squirrel left atrium) was, in fact, MHC-α (Fig. 2A) and this antibody did not recognize the MHC isoforms in gray squirrel masseter or temporalis. Furthermore, when samples of masseter, temporalis and left atrium were run on a separating gel with 5% glycerol, the two isoforms were clearly separated from each other (Fig. 1D), indicating the predominant MHC isoform in gray squirrel masseter and temporalis is not MHC-α. Mass spectrometry was next used to assist in the identification of the predominant MHC isoform in the gray squirrel masseter and temporalis. The results strongly suggested that the protein was the masticatory (‘superfast’) MHC isoform (‘MHC-M’; matched with chimpanzee MHC-M, accession number XP 519229, NCBI database, 309 peptides matched, MOWSE Score=5167). The pattern of migration of the predominant MHC band, relative to limb muscle slow-type and fast-type MHC bands, in gray squirrel was very similar to that in two species in which MHC-M has been reported to be expressed [domestic dog (Rowlerson et al., 1983); American opossum (Sciote et al., 1995) (and not shown)]. An immunoblot (Fig. 2A) was run with an anti-MHC-M antibody and the results indicated that the predominant MHC isoform in gray squirrel masseter and temporalis is MHC-M and migrates similarly to MHC-M in dog and opossum.

A very minor, slightly faster migrating MHC band was also observed in gray squirrel masseter and temporalis samples. This band co-migrated with the faster migrating of the two cardiac MHC isoforms. Others have shown that mammalian skeletal slow-type MHC-I and cardiac MHC-β are the same protein (Cuda et al., 1993; Lompré et al., 1984). An immunoblot with an anti-MHC-I/β antibody confirmed the identity of this isoform (Fig. 2C). The amount of MHC-I, relative to total MHC, was greater in the deeper regions of both muscles (not shown).

Others (Rowlerson et al., 1981; Qin et al., 1994; Sciote et al., 1995) have reported that unique MLC isoforms are associated with cat MHC-M *in vivo*. We, therefore, determined which MLC isoforms are associated with MHC-M in gray squirrel masseter and temporalis. The MLC1 and MLC2 isoforms in gray squirrel tibialis cranialis and soleus were identified by standards that were generated by extracting myosin from single muscle fibers (see Materials and methods and supplementary material Fig. S1). The MLC1 and MLC2 isoforms in masseter and temporalis samples had electrophoretic mobilities that were different from fast-type and slow-type MLC1 and MLC2 (Fig. 3). MLC1 and MLC2 in a masseter homogenate were identified by mass spectrometry as the embryonic/atrial isoform of MLC1 (matched with rat embryonic/atrial MLC1, accession number P17209, NCBI database, 44 peptides matched, MOWSE Score=777) and the masticatory (‘superfast’) isoform of MLC2 [matched with the masticatory (superfast) isoform of human MLC2, accession number Q02045, NCBI database, 16 peptides matched, MOWSE Score=399], respectively. Consistent with the mass spectrometry results, MLC1 in gray squirrel masseter and temporalis co-migrated with MLC1 in the left atrium (Fig. 3).

### MHC and MLC isoforms in chipmunks

The MHC and MLC isoforms expressed in chipmunk masseter and temporalis were determined using the same approaches as for gray

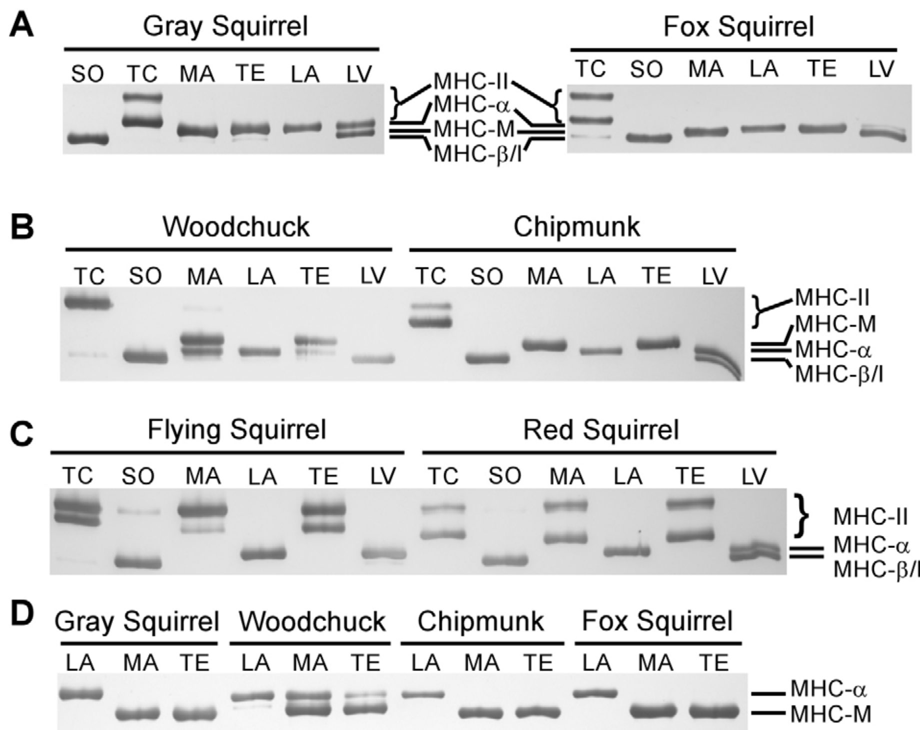



Fig. 1. Electrophoretic separation of myosin heavy chain (MHC) isoforms in Sciuridae species. The MHC region of silver-stained gels, loaded with homogenates of slow-twitch soleus (SO), fast-twitch tibialis cranialis (TC), masseter (MA), temporalis (TE), left atrium (LA) and left ventricle (LV) are shown. (A–C) Separating gels containing 30% (vol./vol.) glycerol. (D) Separating gel containing 5% (vol./vol.) glycerol. Other abbreviations: MHC-II, fast-type MHC; MHC-α, alpha isoform of MHC; MHC-M, masticatory MHC; MHC-β/I, beta (cardiac) or limb slow-type MHC. Note that woodchuck and chipmunk MHC-M migrates slower than MHC-α in gels with 30% glycerol, and that the migration order is reversed in gels with 5% glycerol.

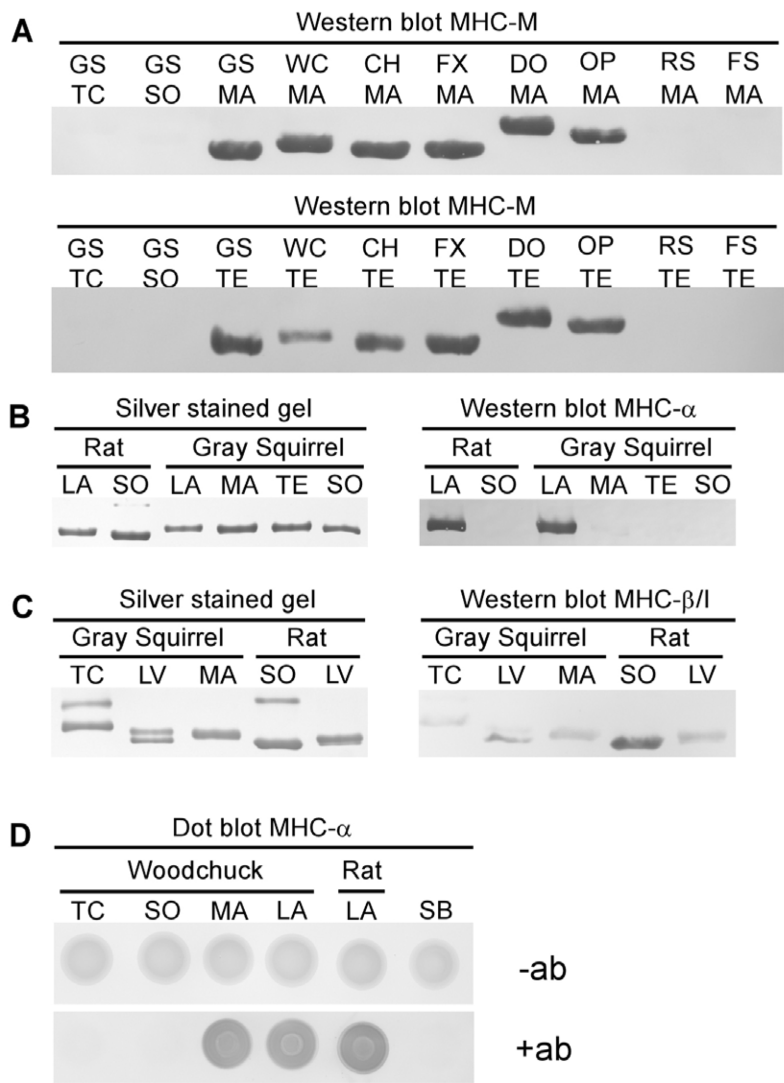



Fig. 2. Immunoblot identification of myosin heavy chain isoforms. Western blots and dot-blots were used to facilitate the identification of masticatory myosin heavy chain (MHC-M), the alpha MHC (MHC-α) and beta (cardiac) or limb slow-type MHC (MHC-β/I) in homogenates of fast-twitch tibialis cranialis (TC), slow-twitch soleus (SO), masseter (MA), left atrium (LA), temporalis (TE) and left ventricle (LV). Other abbreviations: GS, gray squirrel; WC, woodchuck; CH, chipmunk; FX, fox squirrel; DO, dog; OP, North American opossum; RS, red squirrel; FS, flying squirrel; SB, gel sample buffer (negative control). (A) Blots with anti-MHC-M with samples from the masseter (upper) and temporalis (lower). (B) Silver-stained gel (left) loaded with the same samples that were transferred to the membrane for a blot (right) that was probed with anti-MHC-α. (C) Silver-stained gel (left) loaded with the same samples that were transferred to the membrane for a blot (right) that was probed with anti-MHC-β/I. (D) Dot blot loaded with woodchuck and rat samples and probed with anti-MHC-α. Rat samples were used in the blots in B, C and D as positive controls.

squirrel and the results were identical. The predominant MHC isoform in chipmunk masseter and temporalis was identified as MHC-M, based upon gel electrophoretic mobility (Fig. 1B) and immunoreactivity (Fig. 2A). MLC1 and MLC2 in chipmunk masseter and temporalis were identified as MLC1E/A and masticatory isoform of MLC2 (MLC2M), respectively, based upon electrophoretic mobility (Fig. 3). Therefore, the predominant MHC and MLC isoforms in chipmunk masseter and temporalis are identical to those in gray squirrel.

### MHC and MLC isoforms in woodchucks

The myosin subunit isoforms in woodchuck masseter and temporalis were determined next. The predominant MHC isoform in masseter and temporalis was identified as MHC-M, based upon electrophoretic mobility (Fig. 1B) and immunoreactivity (Fig. 2A). Another MHC band in samples of woodchuck masseter and temporalis co-migrated with the predominant MHC band in the left atrium, on separating gels with either 5% or 30% glycerol. A dot-blot (Fig. 2D) was used to test whether the woodchuck masseter expresses MHC-α, the predominant MHC isoform in the atria of most, if not all, mammalian species examined thus far, and the results indicate that this MHC isoform is, in fact, expressed in woodchuck jaw-closing muscles. The isoforms of MLC1 and MLC2 also had electrophoretic mobilities that were distinct from fast-type and slow-type MLC1 and MLC2 (Fig. 3). As was the case for gray squirrel and chipmunk, MLC1 in woodchuck masseter co-migrated with MLC1 in the left atrium (Fig. 3). These results indicate that woodchuck masseter and temporalis express the same MHC and MLC isoforms as do gray squirrel and chipmunk masseter and temporalis.

### MHC and MLC isoforms in fox squirrels

Samples from two fox squirrels were also examined and the results were identical to those obtained from gray squirrel, chipmunk and woodchuck. The masseter and temporalis expressed MHC-M (Fig. 1A; Fig. 2A), as well as MLC1E/A and MLC2M (Fig. 3). MHC-α was not detected in the fox squirrel masseter or temporalis (Fig. 1D).

### MHC and MLC isoforms in flying and red squirrels

The myosin subunits expressed in the masseter and temporalis of three flying squirrels and three red squirrels were also examined. Anti-MHC-M antibody did not recognize myosin in flying squirrel or in red squirrel masseter and temporalis (Fig. 2A), demonstrating that these muscles in flying squirrels and red squirrels do not express MHC-M. The slowest migrating MHC isoforms in the masseter and temporalis of both species co-migrated with the slowest migrating

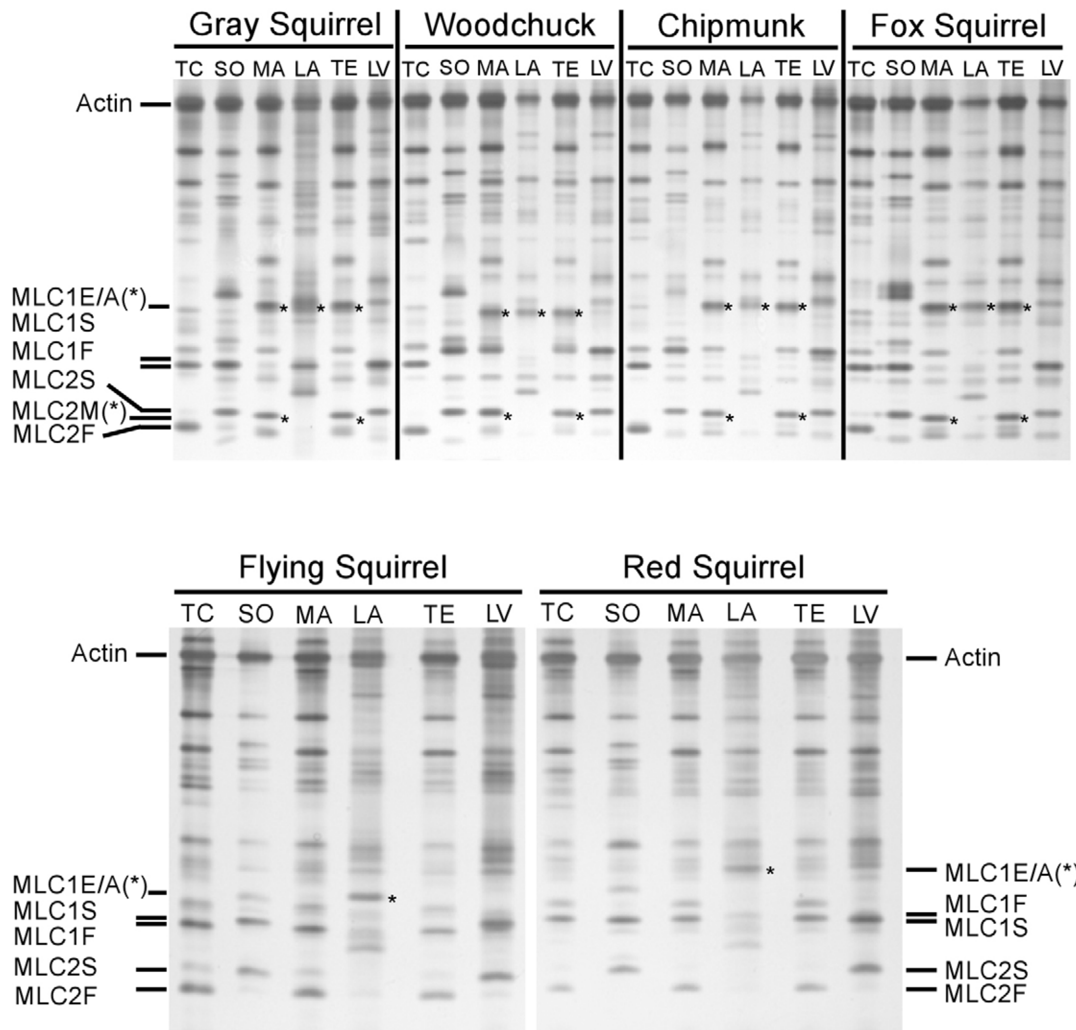
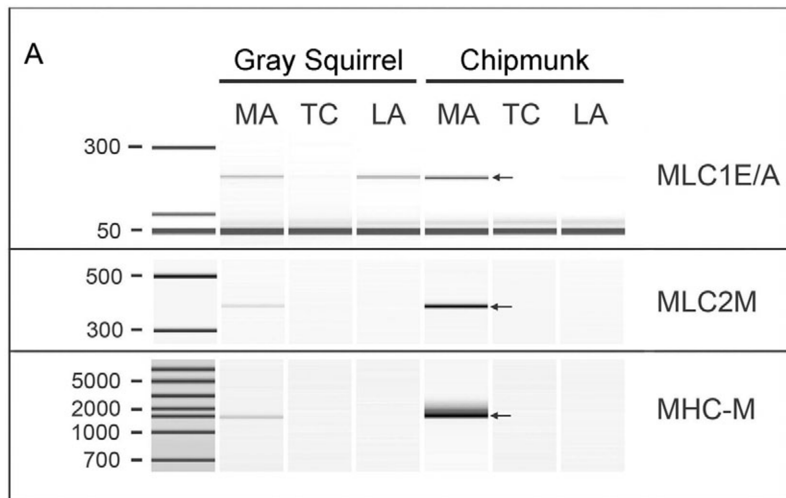



Fig. 3. Electrophoretic separation of myosin light chains (MLC) in Sciuridae species. The lower molecular weight regions of silver-stained gels loaded with homogenates of fast-twitch tibialis cranialis (TC), slow-twitch soleus (SO), masseter (MA), left atrium (LA), temporalis (TE) and left ventricle (LV) are shown. Other abbreviations: MLC1E/A, embryonic/atrial myosin light chain 1; MLC1S, slow-type MLC1; MLC1F, fast-type MLC1; MLC2S, slow-type MLC2; MLC2M, masticatory MLC2; MLC2F, fast-type MLC2. Asterisks mark the right hand edge of MHC1E/A and of MLC2M.

bands in the tibialis cranialis and soleus muscles (Fig. 1C) and are assumed to be fast-type MHC isoforms that are typically expressed in limb muscles. The fastest migrating MHC bands in red squirrel and flying squirrel masseter and temporalis migrated slightly faster than the fastest migrating MHC bands in the tibialis cranialis (Fig. 1C). The band in the masseter and temporalis samples was more abundant [70±8 and 52±6 (means ± s.e.m.), respectively, percentage of total MHC] in the red squirrel samples, compared with those from flying squirrels (28±8 and 41±1). Therefore, the fastest migrating bands in red squirrel masseter and tibialis samples were analyzed by mass spectrometry, and the results identify the bands as being the IIB isoform of MHC (MHC-IIB) (matched with mouse MHC-IIB, accession number Q5SX39, SwissProt database, 1047 peptides matched, MOWSE Score=7529) in the tibialis, and MHC-perinatal (matched with mouse MHC-perinatal, accession number P13542, SwissProt database, 738 peptides matched, MOWSE Score=5884) in the masseter. MLC1 and MLC2 in the masseter and temporalis of red squirrels and flying squirrels co-migrated with MLC1 and MLC2 in the tibialis (Fig. 3) and are, therefore, assumed to be fast-type MLC isoforms.

**RNA analysis**

Fig. 4 shows results of PCR amplification that targeted MLC1E/A in gray squirrel and chipmunk. 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 gray squirrel and from the masseter of chipmunk. No PCR product was generated using the tibialis cranialis mRNA as template in either species. Fig. 4 also shows results of PCR amplification that targeted MLC2M and MHC-M. PCR products were produced at the expected sizes (386 bp for MLC2M and 1395 bp for MHC-M, predicted from cat MLC2M and MHC-M mRNA sequences, respectively) using masseter mRNAs as templates from both gray squirrel and chipmunk. No PCR products were generated using other muscle tissues mRNAs as templates. The results of sequence alignment are also shown in Fig. 4. The gray squirrel and chipmunk sequences obtained from the MLC1E/A amplicons were 93% homologous to the rat MLC1E/A mRNA sequence. The gray squirrel and chipmunk sequences obtained from the MLC2 amplicons were 88% and 87% homologous to the cat MLC2 mRNA sequence, respectively. The




| B | Sequence Homology | | | | | |
|---|---|---|---|---|---|---|
| | Gray Squirrel (%) | | | Chipmunk (%) | | |
| | MA | TC | LA | MA | TC | LA |
| MLC1E/A (Rat; NM_00119495.1) | 93 | - | 93 | 93 | - | - |
| MLC2M (Cat; NM_001009874.3) | 88 | - | - | 87 | - | - |
| MHC-M (Cat; NM_001009221.1) | 89 | - | - | 90 | - | - |

Fig. 4. Identification of embryonic/atrial myosin light chain 1 (MLC1E/A), masticatory isoform of myosin light chain 2 (MLC2M) and masticatory myosin heavy chain (MHC-M) mRNA sequences in gray squirrel and chipmunk. (A) Electrophoresis results of PCR amplified MLC1E/A, MLC2M and MHC-M mRNA sequences generated from different muscle tissues of gray squirrel and chipmunk. (B) Sequence alignment of the putative gray squirrel and chipmunk MLC1E/A, MLC2M and MHC-M mRNA sequences with the published sequences in other species. Amplicons at expected size (see text) are indicated by arrows.

gray squirrel and chipmunk sequences obtained from the MHC-M amplicons were 89% and 90% homologous to the cat MHC-M mRNA sequence, respectively.

The identification of the predominantly or solely expressed MHC and MLC isoforms in the jaw-closing muscles of the six species that were examined in this study is summarized in Table 1.

## DISCUSSION

### Masticatory myosin is expressed in some rodent species

It is now clear that masticatory myosin is expressed in the order Rodentia, as the results of the present study demonstrate robust expression in the jaw-closing muscles of four Sciuridae species. Approximately 40% of the world's mammals are classified as rodents. The prevailing notion has been that rodents lost the expression of MHC-M when a change in diet occurred during evolution and that MHC isoforms that are typically expressed in limb skeletal muscles replaced MHC-M in jaw-closing muscles. MHC-M is, in fact, expressed in some species (*S. carolinensis*, *T. striatus*, *S. niger* and *M. monax*) of the order Rodentia but not in others (*G. volans* and *T. hudsonicus*) that are remarkably closely related phylogenetically to some of the species that express MHC-M. This is even more striking when considering the significant overlap in the diets of some of the rodent species that were studied. It is not clear if the expression of masticatory myosin in some rodent species is a consequence of evolutionary convergence or if the absence of masticatory myosin in other rodent species that express myosin isoforms typically found in perinatal and adult limb muscles is a relatively recent evolutionary change. Comparative genomic studies should reveal whether masticatory myosin genes remain intact in rodent species in which masticatory myosin expression is absent. Related to these issues, humans are the only primates known to not express MHC-M. Stedman et al. reported that the MHC-M gene in humans is inactive (coding a drastically truncated protein) due to a frameshift resulting from a two-base deletion (Stedman et al., 2004). There are other reports of masticatory myosin being expressed in some, but not other closely related, species. The lesser (red) panda (*Ailurus fulgens*), which has a primarily herbivorous diet, is the only studied species of Carnivora that does not express masticatory myosin (Rowlerson et al., 1983). Kang et al. reported that *Miniopterus schreibersii* is exceptional among the seven species of Chiroptera that they examined in that it does not express masticatory myosin (Kang et al., 1994). Among the eleven marsupial

Table 1. Summary of the predominant myosin heavy chain (MHC) and light chain (MLC) isoforms in jaw-closing muscles

| Species | MHC isoform | MLC1 isoform | MLC2 isoform |
|---|---|---|---|
| Eastern gray squirrel | Masticatory | Embryonic/atrial | Masticatory |
| Eastern chipmunk | Masticatory | Embryonic/atrial | Masticatory |
| Woodchuck | Masticatory | Embryonic/atrial | Masticatory |
| Eastern fox squirrel | Masticatory | Embryonic/atrial | Masticatory |
| Red squirrel | Fast and perinatal | Fast | Fast |
| Southern flying squirrel | Fast and perinatal | Fast | Fast |

species studied thus far (Sciote et al., 1995; Sciote and Rowlerson, 1998; Hoh et al., 2000; Hoh et al., 2006), only kangaroos and wallabies do not express masticatory myosin. These differences might reflect evolutionary adaptations that accommodate acquired differences in feeding styles or foraging strategies. An evolutionary shift in diet, perhaps to take advantage of an alternate food source, might have allowed the genes to become silent or to be lost, if species survival is no longer dependent on their expression. Alternatively, MHC-M and/or MLC2 genes might have acquired mutations in the species in which these proteins are not expressed, forcing a change in diet.

### MLC1E/A is expressed with MHC-M

The results also indicate that the isoform of MLC1 that is associated with MHC-M is not a unique isoform, and is, instead, the MLC1E/A isoform, in the four Sciuridae species now known to express MHC-M. Rowlerson et al. compared the electrophoretic mobility of MLC1 in cat masseter with the mobility of fast-type and slow-type MLC1 isoforms from limb muscle and concluded that unique MLC1 and MLC2 isoforms are associated with MHC-M (Rowlerson et al., 1981). Our results show that the masticatory isoform of MLC2 is expressed with MHC-M in gray squirrel, fox squirrel, woodchuck and chipmunk jaw-closing muscles, consistent with Rowlerson et al. (Rowlerson et al., 1981). However, our results, obtained by using multiple experimental approaches, also show that MLC1E/A is associated with MHC-M, in four Sciuridae species studied. It is possible that the expression of MLC1E/A in adult jaw-closing muscles in these species is due to the lack of a gene encoding a more specialized MLC1 isoform, even though specific isoforms of MHC and MLC2 (i.e. MHC-M and MLC2M) for functions such as mastication or defensive biting are expressed.

The role of MLC1E/A in regulating contraction has been probed in other studies, and the results from these studies might provide insight to understand the significance of the association of MLC1E/A with MHC-M in jaw-closing muscles. Fewell et al. reported that expression of MLC1E/A in the cardiac ventricles of transgenic mice caused an increased rate of myosin-driven thin filament sliding, using an *in vitro* motility assay, as well as increased rates of shortening in isolated ventricular strips and of pressure development and relaxation in the whole heart (Fewell et al., 1998). These results were consistent, as discussed by Fewell et al. (Fewell et al., 1998), with the increased rates of muscle shortening and force production associated with the expression of MLC1E/A in the ventricles of human cardiomyopathic hearts (Morano et al., 1996), as a possible compensatory mechanism. The affinity of MLC1E/A for actin is lower than is the actin-affinity of slow-type MLC1 (Morano and Haase, 1997), possibly allowing more rapid crossbridge cycling and, thereby, providing a mechanism for faster contractile properties in fibers expressing MLC1E/A. This would suggest that MLC1E/A facilitates more rapid contractions in jaw-closing muscles. However, what needs to be considered is the gain (or loss) of function associated with the substitution of MLC1E/A for other MLC isoforms that would otherwise be associated with MHC-M. The results from Hoh et al. (Hoh et al., 2007) are particularly interesting in this regard. These authors reported that masticatory MLC1 and 2 are associated with MHC-M in some fibers of cat masseter, consistent with Rowlerson et al. (Rowlerson et al., 1981), and with slow-type MHC-I in other fibers of the same muscle, consistent with Sciote et al. (Sciote et al., 1995). Furthermore, Hoh et al. (Hoh et al., 2007) reported that the slow fibers in cat masseter have significantly faster contractile kinetics, compared with slow fibers in limb muscle, which express the same MHC isoform but not masticatory MLC1 or 2. This suggests that MLC1E/A and masticatory MLC1 have similar roles in modulating (increasing) contractile kinetics. Therefore, expression of MLC1E/A may impart greater power output in jaw-closing muscles in some squirrel species, which could provide an advantage for nut opening.

### Myosin isoforms and rodent feeding styles

There is a lot of dietary overlap among the six Sciuridae species in this study (Whitaker and Hamilton, 1998). All of the species, except woodchucks, consume a large number of nuts, along with a variety of seasonally available items, such as seeds, flowers, insects, bird eggs and nestlings. The woodchuck diet consists more of bark, buds and twigs of trees, especially when food is sparse in the spring, along with grasses and several farm crops. Therefore, an association of myosin isoform expression in jaw-closing muscles with food type or consistency, among the species studied, is not clear. Wells-Gosling (Wells-Gosling, 1985) provided a description [also discussed by Whitaker and Hamilton (Whitaker and Hamilton, 1998)] of differences in nut opening/cracking patterns among several rodent species. Southern flying squirrels and red squirrels create mostly single openings of shells to access the nutmeat whereas white-footed mice create numerous small perforations of the shell to facilitate accessing the meat. Gray squirrels and fox squirrels, however, essentially obliterate the shell. Our results are fully consistent with these differences in feeding styles, given that masticatory myosin appears to impart high force generating ability to those muscle fibers in which it is expressed (reviewed by Yamaguchi, 2007; Reiser and Bicer, 2007; Toniolo et al., 2008) and it is likely that high forces are needed to break the shell into many small pieces. It is well known that, following the introduction of gray squirrels to England in the late 1800s and during their subsequent burgeoning population increase, the resident red squirrel population sharply declined, as initially described by Middleton (Middleton, 1930) and subsequently studied by several other investigators (e.g. Reynolds, 1985; Teangana et al., 2000). A causal relationship between these two population swings has not been definitively determined. Nevertheless, our results suggest that gray squirrels brought with them the expression of masticatory myosin with an enhanced ability to break hard-shell nuts for food acquisition, possibly contributing to their dominance, at least in some regions.

The results also demonstrate that MHC-$\alpha$ is expressed, along with MHC-M and MHC-$\beta$, in jaw-closing muscles of the woodchuck (*M. monax*). Widmer et al. reported that MHC-$\alpha$ is present at a very low level in adult mouse masseter (Widmer et al., 2002). Expression of MHC-$\alpha$ has also been reported in rabbits (Bredman et al., 1991; d'Albis et al., 1993; Sciote and Kentish, 1996) and in humans (Bredman et al., 1991; Pedrosa-Domellöf et al., 1992; Sciote et al., 1994; Stal, 1994; Korfage et al., 2000), with the level in both being relatively low. However, MHC-$\alpha$ is almost exclusively expressed in kangaroo masseter (Hoh et al., 2000). In a more recent study, Hoh et al. examined the relative numbers of fibers expressing MHC-M or MHC-$\alpha$, the latter co-expressing MHC-$\beta$, in the masseter of seven other marsupial species (Hoh et al., 2006). The relative number of MHC-$\alpha/\beta$ co-expressing fibers decreased with the amount of dietary vegetable matter among these seven species. Although samples from only one woodchuck were examined in the present study, the results suggest that woodchuck jaw-closing muscles have a relatively high level of MHC-$\alpha$ in the masseter and temporalis, compared with mice. The woodchuck diet is more herbaceous (grasses, clover, etc.) compared with the very mixed diet of mice (seeds, small nuts, insects, etc.). The expression of MHC-M, as well as

MHC-α and MHC-β, in woodchucks probably accommodates a diet that includes hard plant material that is likely to be less prevalent in the natural diet of mice.

Another unexpected result is the relatively high level of MHC-perinatal in the jaw-closing muscles of red squirrels, identified based upon results from mass spectrometry and an electrophoretic mobility that is different from MHC isoforms in fast and slow limb muscles. The same electrophoretic mobility difference was observed in jaw-closing muscles of flying squirrels and, by inference, it appears that this species also expresses MHC-perinatal in the masseter and temporalis. All of the animals in the present study appeared to be adults, based upon body mass. The relatively high level of MHC-perinatal in the masseter and temporalis of red squirrels and its apparent presence in the same muscles in flying squirrels, at lower levels, suggest that this isoform is stably expressed in adults. It is well known that developmental isoforms of MHC are expressed in mammalian adult jaw-closing muscles (d'Albis et al., 1986; Butler-Browne et al., 1988; Soussi-Yanicostas et al., 1990; Bredman et al., 1992; Sciote et al., 1994; Stal et al., 1994; Monemi et al., 1996; Eason et al., 2000; Korfage et al., 2000; Widmer et al., 2002), as well as adult extraocular muscles (Wieczorek et al., 1985; Sartore et al., 1987; Rubinstein and Hoh, 2000; Lucas and Hoh, 2003; McLoon and Christiansen, 2003; Bicer and Reiser, 2009) and laryngeal muscles (Toniolo et al., 2005; Mu et al., 2004). In general, the levels of the developmental myosin isoforms in adult mammalian craniofacial muscles, described previously, are much lower than what is observed in red squirrel masseter and temporalis. Additional studies are needed to determine if muscle fibers that express MHC-perinatal in jaw-closing muscles of adult rodents have unique contractile properties that might serve specific feeding styles.

### Masticatory myosin associates with very different vertebrate dentition patterns

Attempts are often made to associate biting and/or chewing characteristics with specific patterns of dentition (or tooth formulae – numbers of upper and lower incisor, canine, premolar and molar teeth), as found among members of Carnivora that express masticatory myosin and hunt and that typically have large canine teeth for piercing prey, carnassial teeth for tearing flesh and large molars for crushing bones. Sharks and crocodiles, which also express masticatory myosin, also have teeth that are specialized for capturing and holding prey while rapid whole-body rolling (crocodile) or rapid head turning (sharks) has a tendency to dismember prey, with subsequent ingestion and swallowing of large food items. Rodents share a very similar dentition pattern, which is markedly different from the patterns associated with high bite forces in other animals, which are presumed to be generated by fibers expressing masticatory myosin. Most rodents, including all of the species in this study, have 20–22 teeth whereas many members of Carnivora with large body mass (e.g. domestic dog, coyote, gray wolf and red wolf, red fox and black bear) have 40–42 teeth, although felids typically have fewer (28–30) teeth (Whitaker and Hamilton, 1998). The fact that masticatory myosin is expressed in only some rodents raises the question of how essentially the same dentition pattern is functionally coupled to the presence of limb-type and perinatal myosin isoforms in some species and masticatory myosin in other species. Furthermore, it is commonly thought that the characteristics of the entire masticatory apparatus (e.g. mandible size and shape, dentition patterns, morphology of individual teeth, myosin isoform expression) in individual species have evolved to accommodate specific and highly divergent feeding styles and behaviors. Our results, however, indicate that strikingly different (almost non-overlapping) myosin isoform expression patterns are found among Sciuridae species, which share identical or nearly identical dentition patterns and mandible shape and size, the latter scaling closely with whole-body size. Additional studies are needed to determine the physiological significance of the different myosin expression patterns, including masticatory myosin, isoforms normally expressed in adult limb muscles, and developmental isoforms in jaw-closing muscles of rodents.

## LIST OF ABBREVIATIONS

| | |
|---|---|
| MHC | myosin heavy chain |
| MHC-α | alpha isoform of myosin heavy chain |
| MHC-β/I | beta isoform (slow-type) of myosin heavy chain |
| MHC-IIB | IIB isoform of myosin heavy chain |
| MHC-M | masticatory isoform of myosin heavy chain |
| MLC | myosin light chain |
| MLC1 | myosin light chain 1 |
| MLC1E/A | embryonic/atrial isoform of myosin light chain 1 |
| MLC2 | myosin light chain 2 |
| MLC2M | masticatory isoform of myosin light chain 2 |

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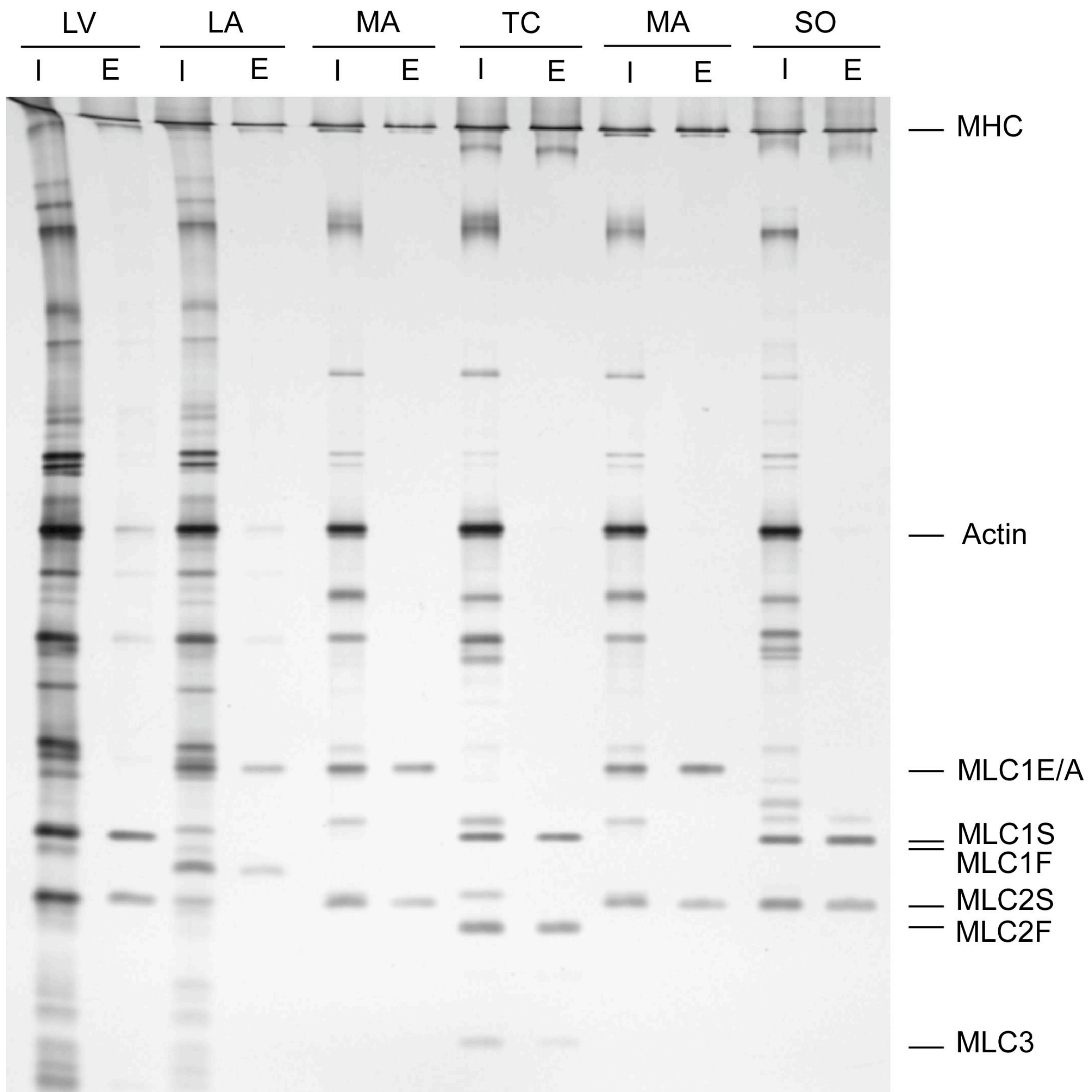
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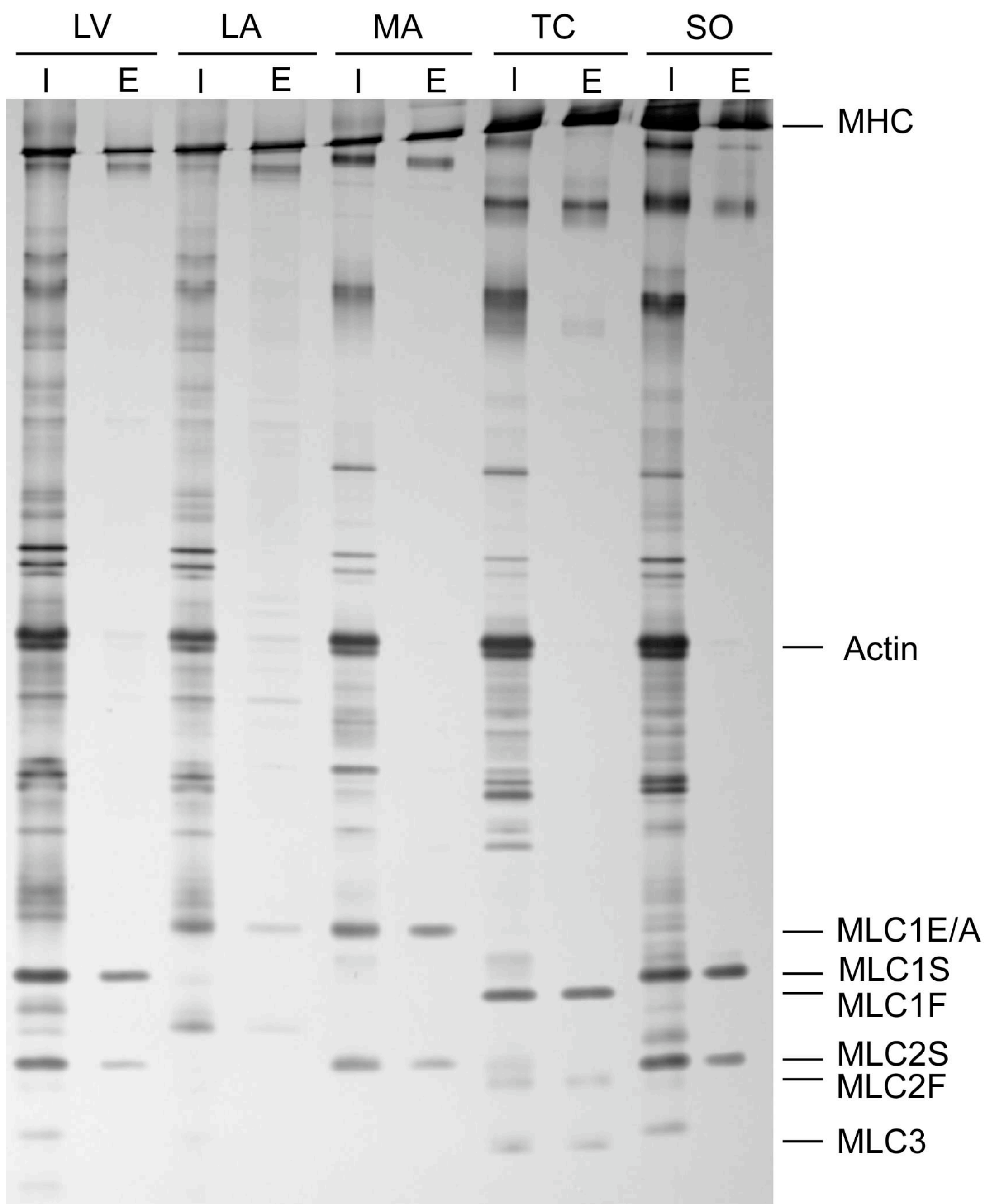
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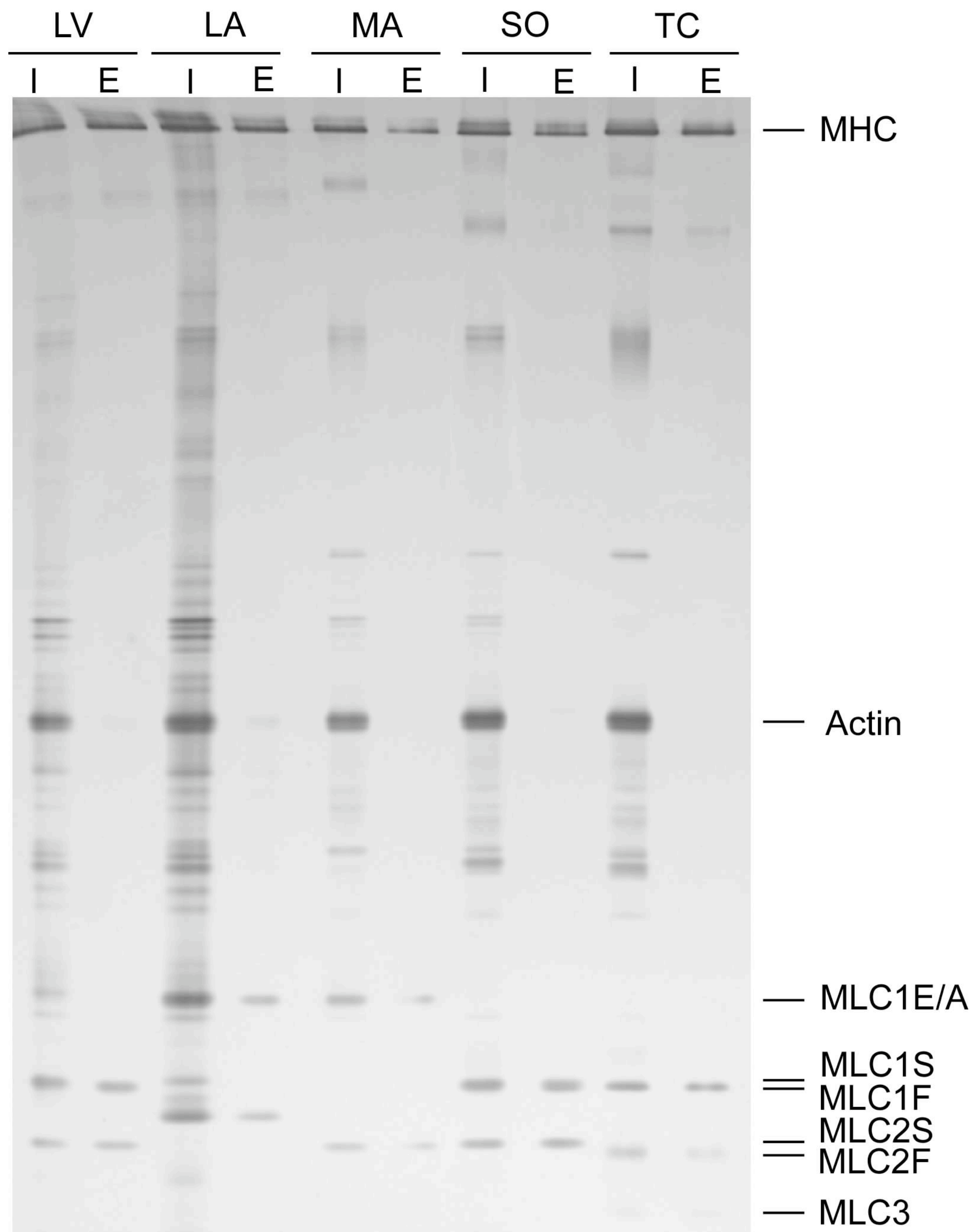
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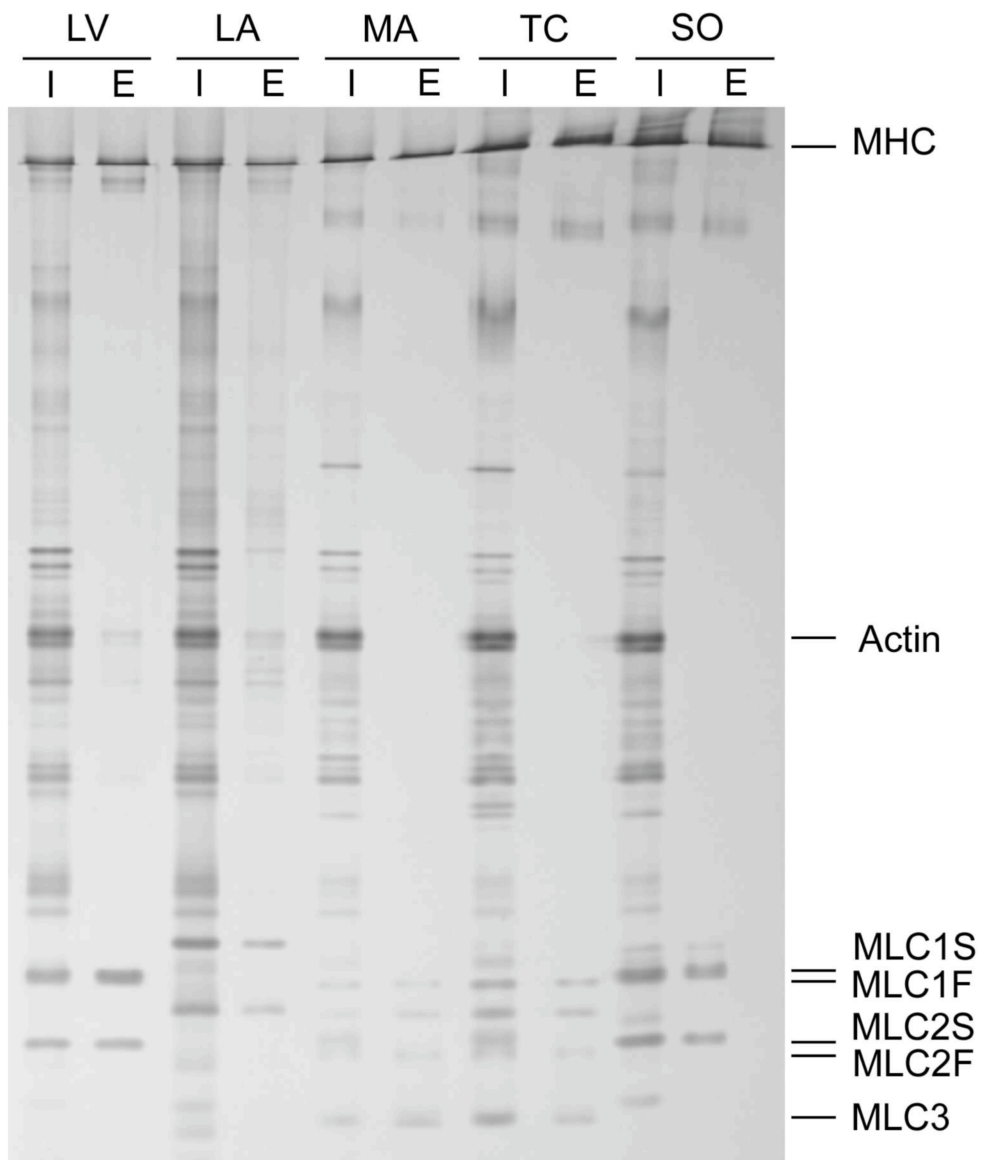
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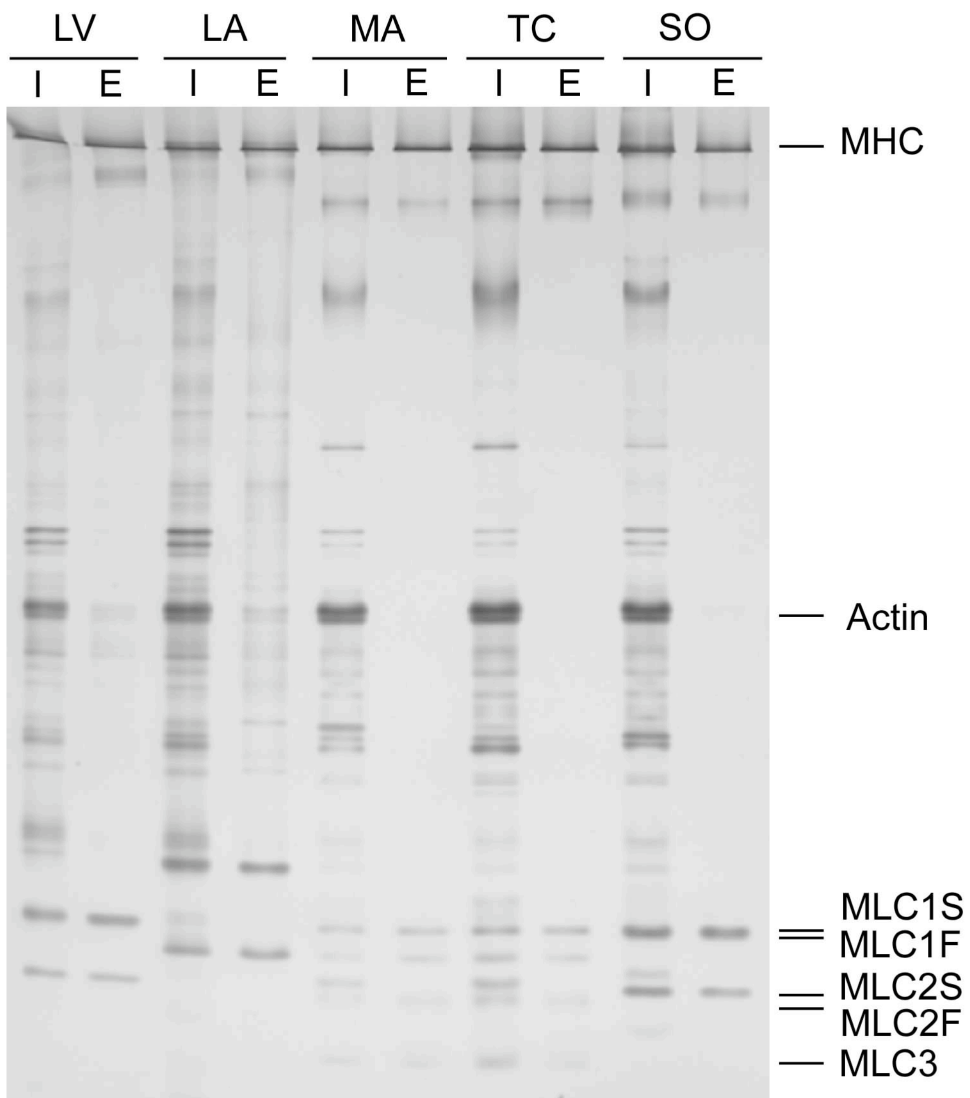
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