Repeated evolutionary turnover of vertebrate skeletal muscle myosins

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Keywords: myosin, vertebrates, evolution, molecular evolution, skeletal muscle, gene family evolution, RNA-Seq, protein structure

Abstract

Myosin heavy chain proteins are essential for muscle contraction and nearly every physiological function in animals, but their diversity and evolution outside mammals is largely unknown. We comprehensively model the evolutionary history of over 1100 heavy-chain myosins. We find that skeletal muscle myosins are located in a conserved tandem gene array in all vertebrate species, but repeated gene duplication-loss turnover has surprisingly led to an independently evolved set of core skeletal muscle myosins in each major vertebrate group. Despite these separate derivations of these myosin subfamilies, each major vertebrate group exhibits consistent tissue-specific patterns of subfamily expression and specialized myosin subfamily expression in extreme muscles. Our results show that muscle evolution across vertebrates is not based in conserved orthologous motor myosins, as might be expected for such a core structural protein family. Instead, we find that skeletal muscle myosins have evolved as a shifting cluster of genes that is constantly changing and diversifying to balance the need to maintain core physiology, while innovating new physiological possibilities.

1. Introduction

Physical movement is fundamental for animal survival and reproduction, helping individuals find food, evade predators, mate successfully, and parent offspring. Myosin heavy chain (MYH) proteins actuate much of this movement by endowing skeletal muscles with contractile properties. Physiological studies have repeatedly suggested that MYH proteins are conserved across taxa suggesting minimal evolutionary change in these core molecules [1-5]. In contrast, molecular genetic studies have found extraordinary diversity in MYH proteins broadly, and have long hinted at a more complex underlying process for the adaptive evolution of skeletal muscle MYHs involving repeated evolution of distinct sets of MYHs [6-14]. The fundamental tension between diametric perspectives of genetic conservation and repeated divergence can only be resolved by comparing MYH gene sequences and expression across a large group of unrelated taxa. Such work promises to reveal how MYH proteins have diversified historically, how they contribute to contemporary performance traits, and the central question of whether myosin evolution is dominated by slow conservative evolution or repeated divergence and innovation.

Vertebrate skeletal muscle MYH proteins pull against actin filaments to generate a power stroke that creates force by shortening or lengthening the muscle cell. MYH proteins initiate this process by hydrolyzing ATP into ADP to power actin-myosin binding kinetics [15-18]. Even subtle amino acid sequence variation can confer major alterations to MYH molecular properties [19-22]. The presence of impactful variation creates ample opportunity for selection to drive evolutionary changes in muscle performance.

Mammals have a set of sarcomeric MYH proteins known to have distinct expression patterns and molecular properties that have contributed to the past categorization of “fast” and “slow” contractile speeds [4,23-25]. MYH3 and MYH8 are primarily expressed in embryonic and neonatal tissues, with only minimal expression in some specialized adult muscles [26,27]. Adult skeletal muscles typically express MYH1, MYH2, MYH4 that are exclusively expressed in skeletal muscles, along with MYH7 that has roles in both cardiac and skeletal muscles (Fig 1A)[26]. Larger mammals tend to use a combination of MYH1, MYH2, and MYH7 in their skeletal muscles, but smaller-bodied rodents have been shown to primarily express MYH4 [28,29]. The molecular properties of these MYH proteins have been shown to be distinct with regard to ATP efficiency and mechanical movement, which connects the different MYH molecular properties to the performance properties of the muscles [21].

Current conventional wisdom holds that other vertebrate genomes contain MYHs that are orthologous in origin and function to those observed in mammalian genomes. This belief most likely stems from the observation that distinct muscle fiber types with notable performance differences have been observed in a variety of nonmammals [30,31]. For example, “superfast” muscles in vertebrates support this idea, where extremely rapid contraction-relaxation speeds are associated with distinct MYH compositions [14,32]. The presence of distinct MYHs in these tissues has been noted, though their genomic origins and relatedness to those observed in mammalian fibers have not been assessed in detail [33,34]. MYHs across vertebrates may be genetically and functionally distinct in ways that have yet to be fully described [8]Fig. 1A;. This suggests a different relationship between physiological evolution of vertebrate muscles and the molecular evolution of MYHs where evolution of individual MYH sequences and evolution of the relative expression of MYHs within muscles are joint, interrelated processes.

Here, we conduct a comprehensive study of the origins and expression of MYH genes across chordates by analyzing over a thousand MYH sequences and dozens of skeletal muscle expression profiles. We use our results to test whether MYH evolution is more generally conservative or diversifying. We find that vertebrate genomes encode sets of MYH proteins that are more diverse in gene number, genomic arrangement, and molecular structure than has been described previously. Our results conclusively demonstrate that primary skeletal MYH proteins in each major group of vertebrates evolved separately with respect to each other and lack one-to-one evolutionary relationships. These separate MYH diversifications precludes the possibility of homologous functional roles and suggests convergent pressures where similar muscle properties and functions occur in separate groups. Finally, the tissue-specific expression of MYHs in both ordinary and extreme-performance muscles points to a strong and generalized connection across vertebrates between MYH gene functional properties and muscle performance. Most broadly, this expanded evolutionary history of sarcomeric MYHs shows that skeletal myosins show lability despite being fundamental to animal reproduction and fitness, demonstrating a dramatic example of how core genes simultaneously maintain homeostasis while adapting new innovations.

2. Materials and methods

(d) RNA-Seq Data

Lonchura striata syrinx raw RNA-seq sequences are available from NCBI SRA under BioProject #. All other expression data were obtained from public sets available on NCBI. The accession numbers to these sets are provided in Data S5 and paper DOIs are provided when available

(e) Compiling Amino Acid Sequences

We compiled a sample of amino acid sequences by searching within the NCBI reference genomes of select species for myosins or their syntenic neighbors (Data S1 and Data S2). For species that did not have well annotated reference genomes, we sought out sequences by using NCBI's Basic Local Alignment Search Tool for proteins (BLASTp) or expanded searches to ENSEMBL. This resulted in a set of 1397 sequences prior to filtering and quality control.

(f) Sequence Alignment and Phylogenetic Reconstruction

We filtered our dataset to remove sequences that were partial or of poor quality. We also made small manual edits as needed to remove regions prior to start codons, retained introns, and extended repetitive ends erroneously retained on the c-terminus (see Data S4 for a description of these edits). After filtering, our dataset included 1203137 sequences from 1192 vertebrate species (Data S1 and Data S2). We aligned these sequences using MAFFT's L-INS-i method and created phylogenetic trees using RAXML-ng with the QMaker PFAM model of protein evolution with gamma-distributed rates [36,37]. This was done with the following options:

```
raxml-ng --all --msa ALIGNMENT.fa --model Q.PFAM+G
```

RAXML-ng generates support values by default using the Felsenstein Bootstrapping protocol. Bootstrap replicates were generated until the majority rule extended (MRE) threshold was surpassed, up to a maximum of 1000 replicates.

(g) Phylogenetic Relationships Between Myosins

We determined evolutionary relationships between myosins using both phylogenetic reconstruction and amino acid sequence analysis. First, we grouped phylogenetically monophyletic groups to identify clades of independently diverging myosins. We additionally compared sequence similarity by calculating the pairwise difference between amino acids sequences (Fig. S1). To do this, we used a custom Python3 script to count the pairwise percent distances in amino acids. The numerator was the count of all amino acid sites without a gap in both sequences that were the same amino acid. The denominator was a count of all amino acid sites without a gap in both sequences. Additionally, we computed a multidimensional scaling plot (MDS) from the same pairwise distances using the Python3 *mds* function from the *scikit-sklearn* module (Fig. S2).

We used this combined data to make the best educated hypothesis of evolutionary relatedness between myosin sequences. To differentiate between non-orthologous myosins, we renamed myosins from several animal clades to better represent their evolutionary history. These names are described in detail in Supplementary Table S1, and further detailed in the Supplementary Text.

(h) RNA Expression Quantification and Determination of Differentially Expressed Genes
We mapped RNA expression data to NCBI reference genomes using STAR and quantified expression with featureCounts [38,39]. We began by preparing genome directories using the following options:

```
STAR --runMode genomeGenerate --genomeDir ./GENOMEDIR --genomeFastaFiles
GENOMEFILE.fna --sjdbGTFfile GTFFILE.gtf
```

We then acquired SRA data using FasterQ from the Sequence Read Archive (SRA) Toolkit and trimmed the reads by using the following command:

```
fasterq-dump SRA_ID --p --W --split-3
```

We then used STAR to align expression data against the prepared genome using:

```
STAR --genomeDir ./GENOMEDIR --readFilesIn SAMPLES.fq
```

To quantify expression data, we used featureCounts using the following command:

```
featureCounts --p --a GTFFILE.gtf --o OUTPUT.txt STAR_OUTPUT.bam
```

We transformed the raw read counts into CPM (counts per million) by dividing the counts for each gene by the total number of counts per sample and multiplying by one million. Because myosins are often very similar in sequence and sometimes differ by only a few amino acids, we removed multi-mapped reads. This means that RNA expression depicted in our data is likely lower than is actually present within the tissue, but more accurately represents the diversity and relative abundance of transcripts.

For Sika, we quantified RNA expression using *kallisto* [40]:
```
kallisto quant -t 64 --index $INDEX -o $OUTPUT $FASTQ1 $FASTQ2
```

To identify patterns of tissue specificity and changes in transcript abundance, we quantified and log2 maximum expression [41]. We considered genes with $\tau > 0.75$ to exhibit high tissue-specific expression.

(i) Calculating Conservation Score, Pairwise Distances, and Amino Acid Diversity

To determine areas of high and low conservation, we calculated conservation scores based on an alignment of 624 sequences from the tetrapod core cluster (Data S6, Data S7). Conservation scores were obtained from JalViewJS using the Analysis of Multiple Sequences (AMAS) method [51,52].

We were additionally interested in understanding instances of gene conversion or nonhomologous cross over. To identify potential regions of interest, we compared sequences we suspected of having evidence of these events to suspected orthologous sources in closely related species. We calculated pairwise distances along a sliding window (P-distance) by quantifying the proportion of nucleotide sites that differ between compared sequences (excluding gap and "N" positions when present). Distances were computed across the alignment, assigning each value to the midpoint of each window to produce a position-specific snapshot of divergence. Window sizes were assigned based on their ability to produce statistically stable P-distance estimates without compromising identification of potential recombination breakpoints.

The primary alignment (Supplementary Data S1) was filtered to four data subsets: (1) 227 sequences of MYH1–4, MYH6–8, and MYH13 from 35 mammal species, (2) 349 sequences of MYH3, MYH6, MYH7, MYH13, and MYH20–26 sequences from 36 bird species, (3) 130 sequences of MYH3, MYH6, MYH7, MYH13A, MYH13B, MYH13C, and MYH45–48 from 12 amphibian species, and (4) 106 sequences of MYH3, MYH52–55 and MYH60 from 6 ray-finned fish species. For each position in the amino acid alignment, the average Grantham's distance for each pair of amino acids at the site was calculated as:

$$\underline{d}_{ij} = \frac{1}{n(n-1)/2} \sum_{i<j}^{\square} G_{x_i x_j}$$

where $\underline{d}_{ij}$ is the average Gratham's distance among all pairs of amino acids at a given position, $G_{x_i x_j}$ is Grantham's distance for a given pair of amino acids from sequences *i* and *j* at position *x,* and *n* is the total number of sequences at the position [53]. Positions with more than one sequence having a gap were scored as zero. Eight sequences with partial missing sequences of >10 aa were excluded. This metric should be higher at aligned positions with both higher amino acid sequence diversity and greater amino acid side chain biochemical diversity. Calculations and plotting were performed with a custom Python3 script.

(j) Determining Loop Lengths and Structure

Alignments of Loop 1 and Loop 2 were extracted from the primary alignment (Supplementary Data S1). We determined the start and stop of each loop based on previous work on humans and chicken myosins [3]. We calculated the ungapped length of these regions (see Data S5) and generated Logo-like plots from a sequence alignment of Loop 2 based on myosin type (Data S5, Fig 4).

3. Results

(a) Core skeletal muscle MYH proteins are not one-to-one related among vertebrate classes.

To create a comprehensive model of sarcomeric vertebrate MYH evolution, we modeled the molecular relationships of 1137 sarcomeric heavy-chain myosin (MYH) amino acid sequences from 113 species from all classes and most major orders of chordates (Fig. 1B). Analysis of the evolutionary change in *MYH* sequences and genomic locations reveals broader patterns of vertebrate evolution and many specific patterns within vertebrate subgroups (see Supplementary Text). We find that all vertebrate sarcomeric MYH proteins are related to a single common ancestor, with cytoskeletal *MYH11* as an outgroup. Across vertebrates, *MYH* gene copy numbers and genomic arrangements are far more variable than have been reported previously (Figs. 1B and 1C)[14].

Present across all vertebrates is a tandem cluster of 1–12 *MYH* genes, which has maintained the same syntenic context throughout vertebrate evolution) . This cluster generally includes the core skeletal muscle *MYH* genes (which have historically been referred to as the "fast-type" *MYH* genes; but see Discussion) in each major group of vertebrates (Fig. 1C. While phylogenetic, pairwise distance, and MDS

analyses show that embryonic MYH3 and specialized/amphibian MYH13 are related across major groups, core skeletal MYHs do not have one-to-one relationships between any two major vertebrate groups (Fig 1B; Supplementary Figs. S1 and S2)[8]. Instead, each group's present core MYHs descend from a group-specific common ancestor (Figs. 1B, 1D, and 2) [14]. As a consequence, each major group of vertebrates has a set of *MYH* proteins that have evolved and diversified separately. Therefore, each vertebrate group's set of MYHs would change in response to their separate evolutionary pressures. Within each group, we also identified clearly conserved and previously unidentified *MYH* subtypes. This discovery prompted us to establish a new classification for non-mammalian subtypes *MYH20–MYH60* to promote discussion of their distinct functional roles and evolutionary histories (Fig. 1B, 1C; Supplementary Tables S1 and S2).

Core skeletal cluster *MYH* genes apparently evolve via tandem gene gain and loss, where each group's present MYH proteins are the result of gene duplications and deletions that all occurred after divergence from other groups (Figs. 1D and 2). The core skeletal cluster is a gene cassette consisting of an uninterrupted tandem array of genes that are all encoded on the same strand and by the same number of exons. We frequently observed instances of recent duplicates (>99% similar) and inactivated and partial pseudogenes. The appearance of these features in several independent lineages indicates repeated instances of gene gain and loss via unequal crossing over as the primary genetic mechanism. We further observed sequence homogenization through gene conversion, biased nucleotide content shifts, and intergenic recombination, but the evidence indicates these are lesser and rarer factors in driving cluster evolution in the long term (Supplementary Figs. S3 and S4, Supplementary Text). Amphibians, lungfish, and many ray-finned fishes have additional translocated individual MYHs or tandem clusters whose sequence relationships indicate they are derived from the core skeletal cluster (*MYH47, MYH48, MYH55* subtypes). These other tandem clusters also show expansion, contraction, and pseudogenization that indicates evolution under the same process as the core cluster. We conclude that this core skeletal MYH cluster as a whole has persisted throughout vertebrate history as a conserved gene cassette, but the specific individual genes in each cluster have gradually been replaced through duplication and loss. Additionally, translocated duplicates have established alternate clusters in certain vertebrate groups.

(b) Vertebrates express their separate MYH protein sets in muscle-specific combinations.
Expression patterns of sarcomeric MYHs have largely been studied in mammals, where correlations between MYH expression and muscle performance are commonly observed [4]. However, the findings described above that other vertebrate groups have independently evolved sets of MYHs provokes the question: do other vertebrate groups exhibit patterns of muscle-specific expression of their various MYH protein sets? Knowledge of the individual and collective patterns of expression are a window into the potential performance properties of muscles in the present and the past evolutionary regimes that have shaped them. To address this, we quantified whole RNA-Seq data sets from diverse vertebrate species where multiple muscles were available (Fig. 3). We selected cases where we could analyze locomotor muscles, specialized muscles, and extreme performance muscles. In mammals, both human and sika deer show expression of *MYH1*, *MYH2*, and *MYH7* in major locomotor skeletal muscles (Figs. 1A and 3A; Supplementary Fig. S5). *MYH* genes were also in the highest 1% of genes by expression level and showed moderate levels of tissue specificity in skeletal muscles. Bat breast muscles expressed *MYH1*, *MYH2*, and the usual cardiac-specific *MYH6*, but the "superfast" laryngeal muscles that generate echolocation signals expressed almost exclusively *MYH4* ([54,55]. A primary locomotor muscle in the harbor porpoise

expressed only *MYH2* out of the core skeletal muscle myosins, corroborating our observation that all cetaceans appear to have pseudogenized *MYH1*, *MYH4*, and *MYH13* (Fig. 3C).

For birds, we focused on two passerine species, zebra finches and golden-collared manakins. Two important muscles that power flight and control wing posture, respectively, showed consistent and differentiated patterns of expression involving multiple MYHs (Fig. 3D, E). In zebra finches, the flight-powering muscle expressed seven of its twelve *MYH* genes at relative expression proportions > 5%, while golden-collared manakins overwhelmingly expressed a single *MYH22*. Notably, this latter species is known for its rapid wing-snap courtship display behavior [56]. The wing muscle that actuates this gestural signal showed expression of both *MYH22* and *MYH25*. Interestingly, this same gene (*MYH25*) is also 92% of the *MYH* expression in hummingbird flight-powering muscle (Fig. 3F). The presence of *MYH25* in two separately evolved extreme muscles strongly suggests the possibility of specialization of this *MYH25* protein. Meanwhile, "superfast" syringeal muscle from Bengalese finches showed strong expression of *MYH13*, as expected from previous evidence in zebra finches (Fig. 3G; Mead et al. 2017). However, we also found substantial syringeal expression of *MYH26*, also the most prevalent myosin in hummingbird tongues (Fig. 3F).

Other vertebrate groups show similar expression patterns. In reptiles, Western Diamondback rattlesnakes show distinct *MYH* expression patterns in three different muscle regions in its body, which govern different types of movement from locomotion to tail-shaking (Fig. 3H) [57]. Across individuals, each segment consistently showed the highest expression of a different *MYH* gene. Notably, the "superfast" caudal rattle-controlling muscles expressed high amounts of *MYH42*, which was absent from the other segments [57,58]. In amphibians, Bornean rock frogs expressed multiple forms of the *MYH13A* subtype in hind limb musculature (Fig. 3I). Amniotes have no more than one copy of *MYH13* that is used primarily in specialized muscles like the larynx and syrinx. However, amphibians have many copies of related protein subtypes MYH13A, MYH13B, and MYH13C. This case study preliminarily confirms that frogs use proteins from the MYH13 subfamily as primary locomotor myosins. Finally, an analysis of yellowfin tuna shows expression of multiple MYH55 proteins in white muscle and several MYH60 proteins from a separate cluster in red muscle (Fig 3E, Supplementary Fig. S).

Collectively, these expression data cases show that MYH proteins are not interchangeable, but instead show tissue specificity and consistent ratios among locomotor and specialized muscles. Tissue-specific MYH expression is a common theme across major vertebrate groups, which is notable given the independent evolution of their MYH gene sets. Intriguingly, this shifting balance of expression of MYH genes broadly observed across vertebrates is complementary with recent evidence that cluster MYH genes are collectively activated by a superenhancer described in mice [59]. In particular, we further show that various specialized and "superfast" muscles predominantly express different myosins, and not a universal "extreme myosin." The tissue-specific combinations of MYH strongly suggest that these proteins have diversified to have different molecular properties, and then combine in muscles to facilitate various muscle performance properties.

(c) Vertebrate sarcomeric myosin diversity is concentrated in two ATP-interacting loops.
To explore the molecular variation of MYH proteins both within each species and across protein subfamilies, we analyzed sequence variation of core skeletal muscle MYHs, MYH3, MYH6, MYH7, and MYH13 in a structural context. MYH sequences are highly conserved within and among subfamilies

across the protein, except two regions in the motor domain (Fig. 4A–C). These variable regions constitute the known loop structures "Loop 1" and "Loop 2. ," which are intermolecular binding sites for the crucial interaction with ATP. Both loops include several positively and negatively charged residues separated by multiple short amino acid segments of variable length (Fig. 4D). Across them, the overall length of the Loop 1 (11–20aa) and Loop 2 (20–31aa) vary by 2- and 1.5-fold, respectively. This length variation stands in sharp contrast to the rest of the MYH protein, where even a single amino acid insertion or deletions are virtually nonexistent except for minor 2–3aa variation in the termini. Furthermore, we found that each species has a set of MYHs with diverse Loop 1 and 2 lengths (Figs. 4E–H). Even recently duplicated subfamilies exhibit variable loop length, such as MYH22A1–MYH22A4 in pigeon (Fig. 4F). This means that, although each group's set of core skeletal MYHs are separately duplicated from a group-specific ancestral protein, their loop lengths have repeatedly evolved variation across all species and in all vertebrate groups (Fig. 4I).

The structure of Loops 1 and 2 ATP-binding loop structural variation helps determine MYH contractile properties, largely governing rate-limiting kinetics of ATP and ADP . Previous investigations have noted sequence variation in Loops 1 and 2 across MYHs and explored how these properties may contribute to differences in sarcomere shortening and overall contractile performance binding [3,60,61]). Thus, we hypothesize that the differences we observe in MYH proteins among muscles and species contribute to variation in muscle performance. Supporting this view, we find that muscles with markedly different performance attributes express different ratios and combinations of MYH proteins. Importantly, these data are consistent with a model where the molecular structure of individual MYH genes is, at minimum, not being homogenized in the ATP-binding loops toward a single optimum. This is especially intriguing in the context of MYH proteins expressed in "superfast" muscles. Despite having a relatively similar performance of rapid contractions with little force generation, each species that maintains these muscles does so by expressing different MYH genes that share no obvious loop configuration properties. Selection for "superfast" muscle performance therefore has likely followed lineage-specific biochemical and biophysical pathways. Furthermore, muscle performance variation is not likely determined strictly by a particular molecular structure or even a particular MYH protein, but instead through the combined molecular properties and expression ratios of several MYH proteins with diverse structures.

To characterize further protein variation, we calculated amino acid diversity for each fully aligned site for core MYHs and MYH6/7/60 subfamilies separately within mammals, birds, amphibians, and ray-finned fish (Fig. 4J)[53]. Out of 1893 sites without gaps, we found that 412 (22%) were completely invariant across all sequences from these four groups with 68% of invariant sites appearing in the head domain. In contrast, 645 sites (34%) exhibited repeated amino acid variation in all four groups, while the remaining 278 (15%), 258 (14%), and 288 (15%) fully aligned sites were variable in three, two, or a single group, respectively. Sites showing variation in all four groups and variation hotspots overall were found in the N-terminal domain, the light-chain myosin binding site on the neck, and midway down the tail (1230–1260). These intersected with several sites of known mammalian MYH7 functional impactful, while pathogenic variants were nearly always invariant across core myosins (Johnson, et al. 2021; Supplementary Table S3). No specific amino acid state was identified as being common to the myosins known to exhibit extreme muscle properties. This concurs with previous hypotheses that the specific impact of individual amino acid changes on MYH fibers or muscle performance as a whole is difficult to predict [3,62]. This analysis highlights that, particularly in the actin- and ATP-interacting myosin head region, MYH is balancing sites of extreme biochemical and structural conservation with sites that are

repeatedly mutating and show variation in multiple independent groups. Therefore, structural variation is maintained in this gene family across long evolutionary distances, but the same regions have repeatedly evolved higher variation, consistent with our characterization of intermixed evolutionary pressures of homeostatic conservation and physiological innovation.

4. Discussion

(a) Skeletal muscle myosins evolve in a balance of functional maintenance and innovation. Understanding the molecular evolution of skeletal muscles is crucial to deciphering their physiological evolution and the evolution of vertebrates as a whole. The lack of one-to-one relationships among individual core MYH proteins between any two major vertebrate groups rejects the extrapolation of mammalian MYH functional identities or fiber type classifications to other vertebrates. emphasize an alternative perspective on skeletal muscle myosins to explain the surprising evolutionary lability of such a core genetic system. Unequal crossing over creates new myosin copies that gradually acquire diverse molecular properties (particularly in the hypervariable Loop 1 and Loop 2) leading to subfunctionalization or neofunctionalization. Specific muscles express MYH copies in specific combinations to achieve differences in performance ability, with such differences likely ranging from subtle to extreme. At the same time, many performance attributes will also require the collective action of several muscles that use a range of expression ratios of various MYH proteins. We therefore propose that MYH evolution proceeds under layers of tandem gene cluster turnover, shifting expression, and changing musculoskeletal design. This dynamic presumably creates the opportunitesy for gradual evolution, with the entire set of myosins being under a collective coselection at the phenotypic level.

(b) An expanded view of MYH diversity prompts renewed consideration of classification

(c) Conclusions

Several other cases of vertebrate gene family tandem cluster duplications (e.g., opsins, venom, immune factors, hormones) often involve antagonistic genetic systems whose diversification is stimulated by a shifting, exogenous factor [75-78]. The lability of MYH protein duplications and losses is therefore particularly striking, since skeletal and cardiac muscles are core, highly interconnected physiological systems presumably subject to substantial endogenous selective constraints. to understand how individual MYH genes contribute to changes in contractile force, velocity, and metabolics [79,80], this investigation establishes the rampant evolutionary turnover of myosins in the evolution of vertebrates and their independent diversifications. This new perspective on both the present diversity and underlying evolution of MYH proteins we hope will stimulate new perspectives, experiments, and discussion of the evolution of vertebrate muscles and motivate renewed investigation of the diversification of other core animal gene families.

Ethics. Procedures involving animal use reflected in this study and not published elsewhere were conducted in accordance with institutional guidelines for the care and use of animals in research. Experimental protocols and animal care were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California of San Francisco.

Data Accessibility. *Lonchura striata* syrinx raw RNA-seq sequences are available from NCBI SRA under BioProject #PRJNA1394504. All other data are available in the supplementary materials, or through NCBI Protein, Nucleotide, or SRA databases at the accession numbers specified therein.

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Funding Acquisition: JBP, MJF, MSB

Conflicts of Interest Declaration. The authors report no competing interests.

Funding. This work was supported by National Science Foundation grant NSF-DEB#2217117 to JBP, NSF-OISE#1952542 and NSF-IOS#2423144 to MJF, and funding from the Howard Hughes Medical Institute to ERS.

Acknowledgements. Thanks to Peri Bolton, Olivia Delgado, Lisle Gibbs, Caroline Kauh, Ellen Weinheimer, Oli Wood for feedback on this manuscript.

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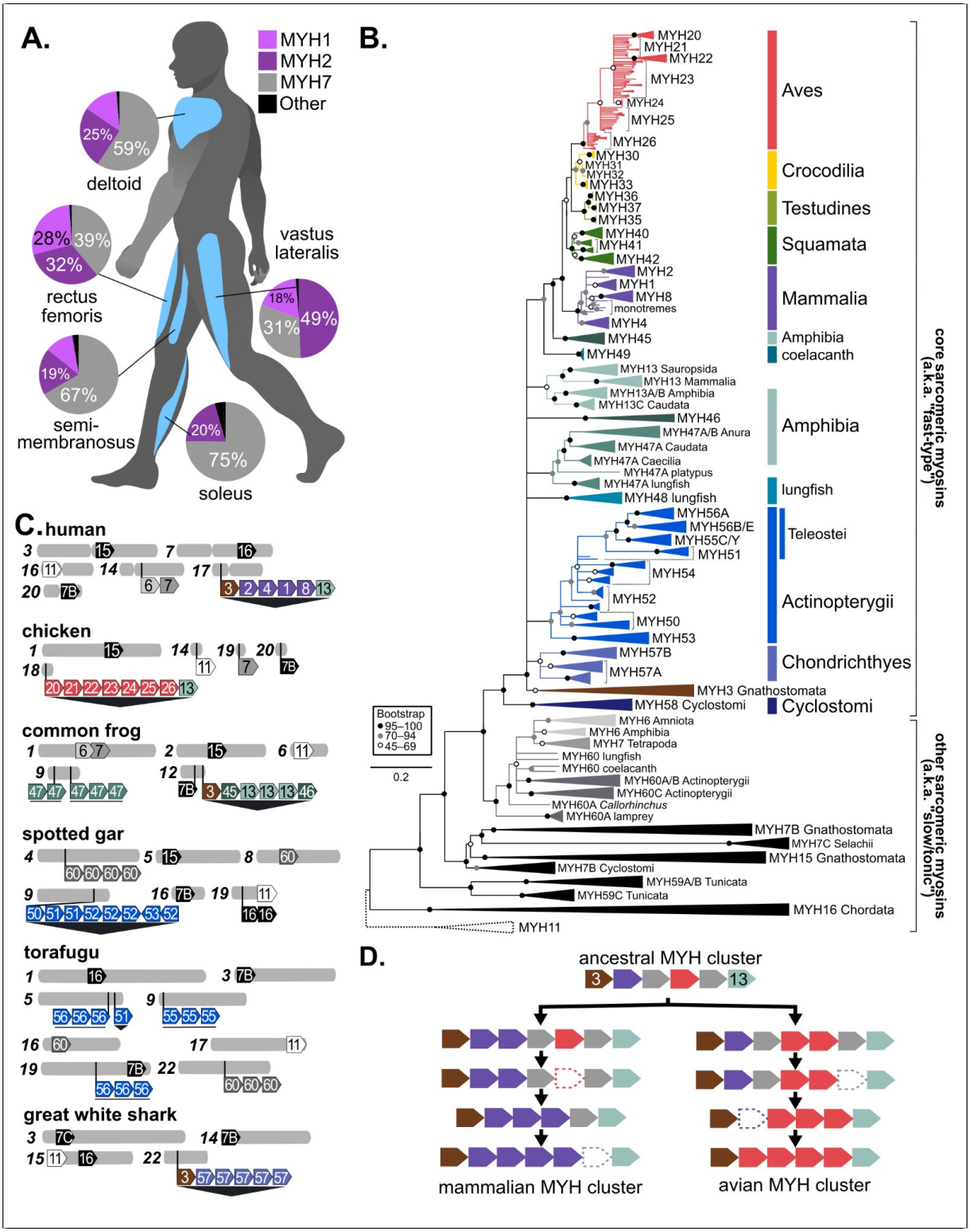
A.
MYH1
MYH2
MYH7
Other
25%
59%
deltoid
28%
39%
32%
rectus femoris
vastus lateralis
18%
31%
49%
19%
67%
semi-membranosus
20%
75%
soleus
B.
MYH20
MYH21
MYH22
MYH23
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MYH36
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MYH35
MYH40
MYH41
MYH42
MYH2
MYH1
MYH8
monotremes
MYH4
MYH45
MYH49
MYH13 Sauropsida
MYH13 Mammalia
MYH13A/B Amphibia
MYH13C Caudata
MYH46
MYH47A/B Anura
MYH47A Caudata
MYH47A Caecilia
MYH47A platypus
MYH47A lungfish
MYH48 lungfish
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MYH56B/E
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MYH51
MYH54
MYH52
MYH50
MYH53
MYH57B
MYH57A
MYH3 Gnathostomata
MYH58 Cyclostomi
MYH6 Amniota
MYH6 Amphibia
MYH7 Tetrapoda
MYH60 lungfish
MYH60 coelacanth
MYH60A/B Actinopterygii
MYH60C Actinopterygii
MYH60A Callorhinchus
MYH60A lamprey
MYH7B Gnathostomata
MYH7C Selachii
MYH15 Gnathostomata
MYH7B Cyclostomi
MYH59A/B Tunicata
MYH59C Tunicata
MYH16 Chordata
MYH11
Aves
Crocodilia
Testudines
Squamata
Mammalia
Amphibia
coelacanth
Amphibia
lungfish
Teleostei
Actinopterygii
Chondrichthyes
Cyclostomi
core sarcomeric myosins (a.k.a. "fast-type")
other sarcomeric myosins (a.k.a. "slow/tonic")
Bootstrap
95–100
70–94
45–69
0.2
C.
human
chicken
common frog
spotted gar
torafugu
great white shark
D.
ancestral MYH cluster
mammalian MYH cluster
avian MYH cluster

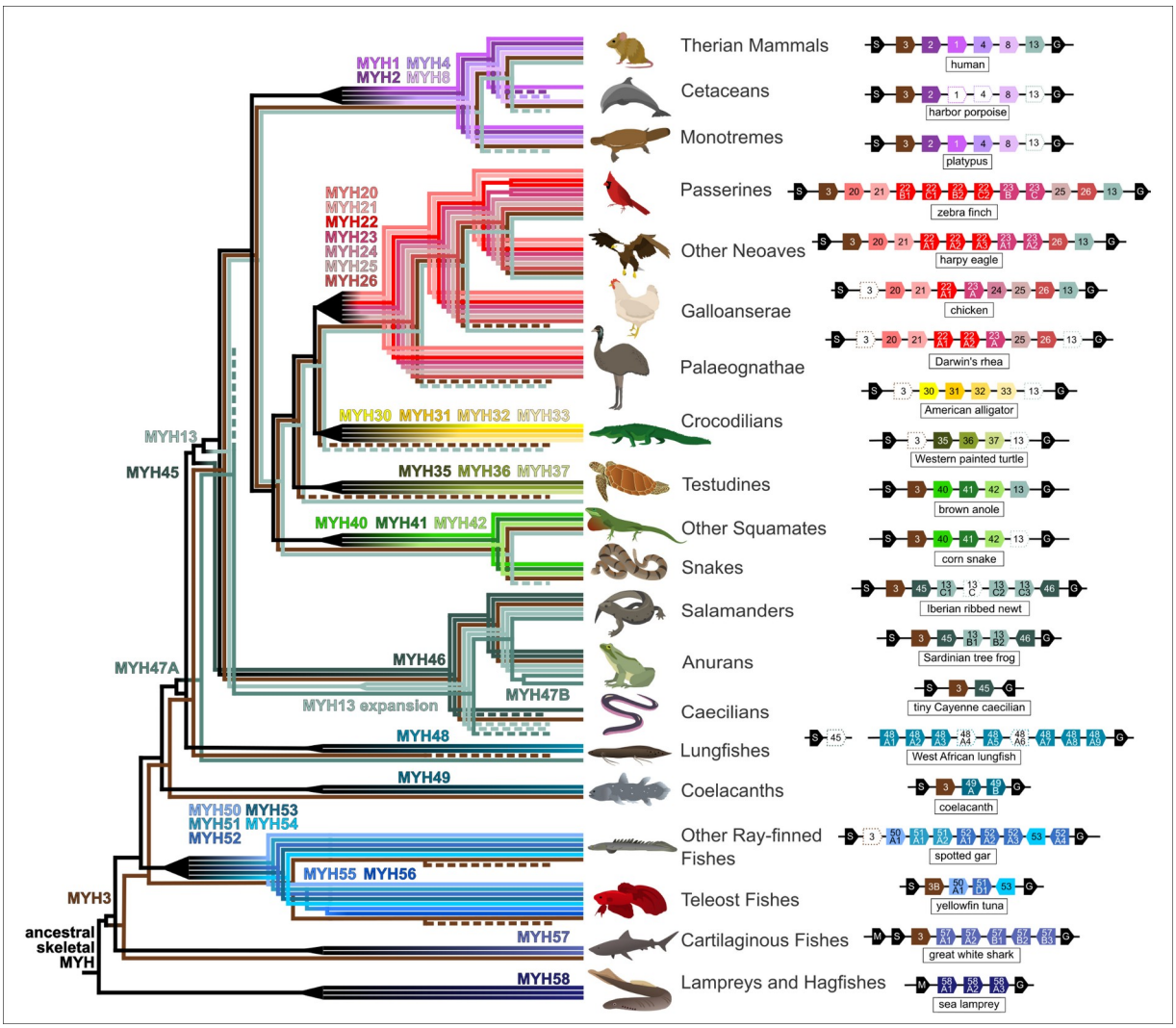

Figure 2. Core skeletal muscle MYH proteins are not one-to-one related among vertebrate classes. Vertebrates show repeated evidence of group-specific myosin subfamily expansion (black triangles) in their evolutionary history (left) and variation in the number of *MYH* genes in their core skeletal muscle MYH cluster (right). Specialized MYH proteins *MYH3* and *MYH13* are related across classes, but pseudogenization through mutational inactivation or partial deletion is also common (dotted outlines). Gene gain-loss in the core cluster occurs in a syntenically conserved locus near *GAS7* (G) and *SCO1* (S) or *MAP24K2* (M). The current lungfish assembly places *SCO1* next to a putatively pseudogenized *MYH45* on a separate chromosome from the rest of the cluster. (Art credit for all drawings: C. Harvey).

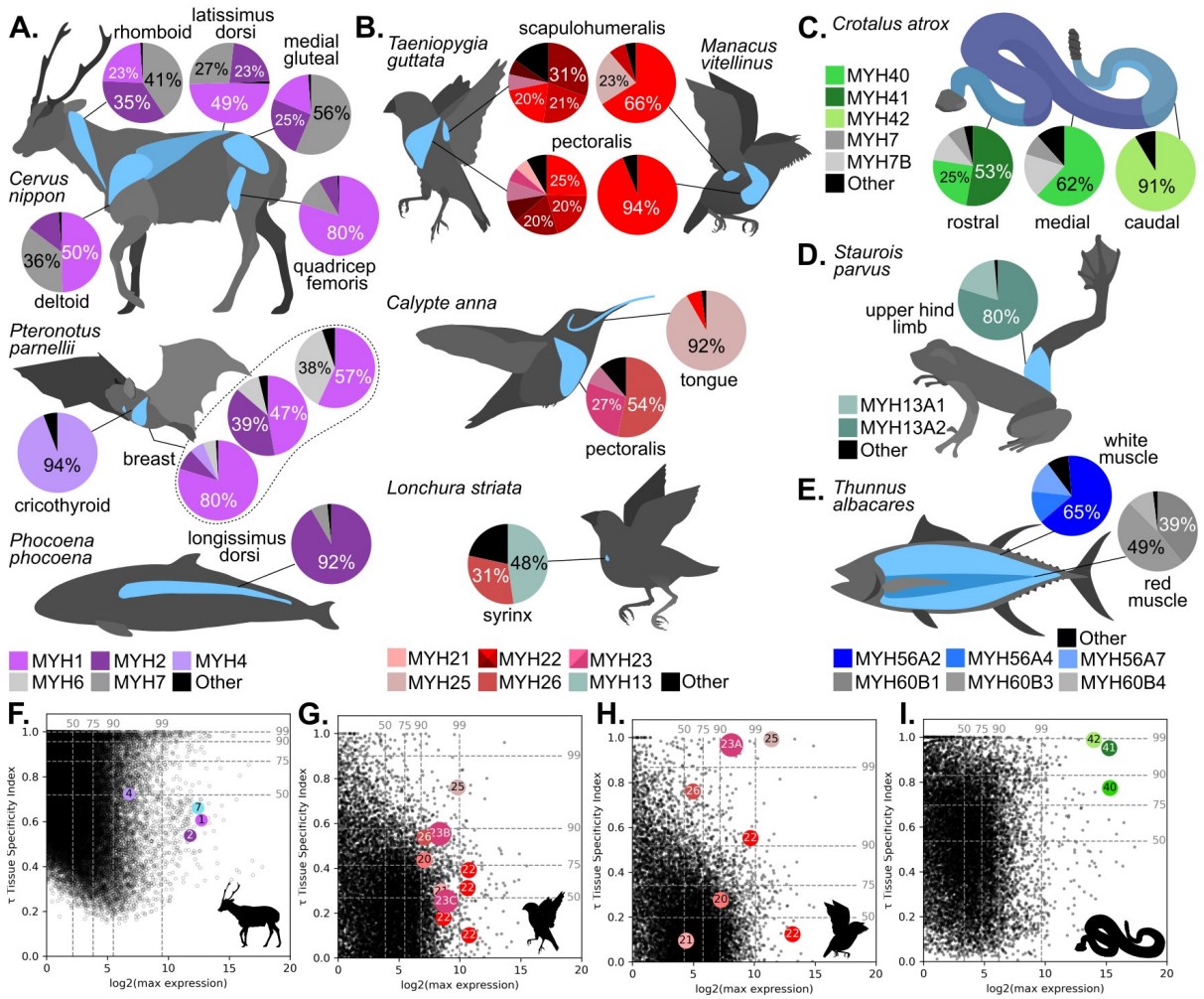

Figure 3. *MYH* genes show variable expression in muscles across vertebrates. The mean proportion of *MYH* expression in each of the highlighted muscles for (A) mammals (sika, Parnell's mustached bat, and harbor porpoise), (B) birds (zebra finch, golden-collared manakin, Anna's hummingbird, and Bengalese finch), (C) Western diamondback rattlesnake, (D) white-spotted foot-flagging frog, and (E) yellowfin tuna. *MYH* genes < 5% relative expression are included as "Other." Breast muscles of Parnell's mustached bat individual samples are depicted with their own pie charts. (F–I) Maximum $\log_2$-expression and tissue specificity index (τ) for all genes with *MYH* genes highlighted as color circles labeled with *MYH* numbers for (F) sika, (G) zebra finch, (H) golden-collared manakin, and (I) Western diamondback rattlesnake. Data distribution percentiles (dashed lines) are shown for each axis. (Art credit for all silhouettes: C. Harvey). See methods and supplementary information for data availability.

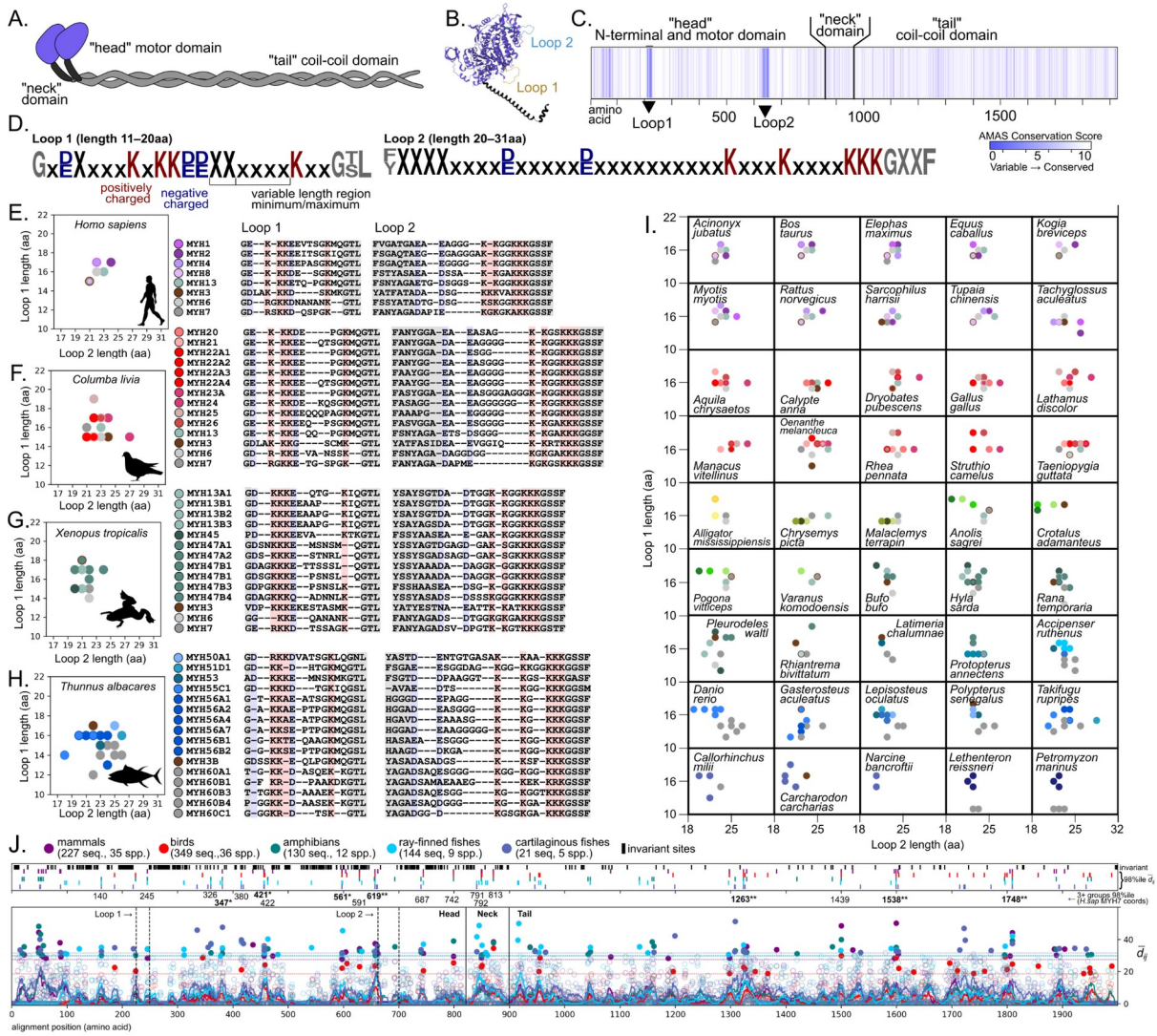

Figure 4. MYH loops and other sites are variable among and within vertebrate species. (A) Myosin protein structural domains shown for a dimerized protein pair. (B) Structure head and neck with Loop 1 and Loop 2 highlighted (PDB#P12882)[63,64]. (C) AMAS conservation scores for each position show that Loop 1 and Loop 2 have the highest rates of variation. (D) MYH Loop 1 and Loop 2 motifs, showing the positive and negative residues and the variable region minimum (X) and maximum (x) lengths observed. (E,F,G,H) Loop 1 and Loop 2 overall lengths create variable combinations for the core sarcomeric and slow sarcomeric MYH proteins in human, pigeon, and western clawed frog, and yellowfin tuna. (I) Loop 1 and 2 combinations for 45 additional vertebrate species show variation in MYH loop lengths is present in species in each major group. Each point represents a single MYH gene, with colors matching subfamilies in Figures 1b and 2. (J) Mean Grantham's distance ($\bar{d}_{ij}$) for all pairs of amino acids at each aligned position in skeletal sarcomeric myosins separately for mammals, birds, amphibians, and ray-finned fish, show amino acid positions that are recurrently variably in multiple groups. Solid graph lines show the mean value for 10aa windows. Positions with more than one gap and MYH7B, MYH15, and MYH16 were excluded. Vertical dashed lines show the Loop 1 and Loop 2 regions, and vertical solid lines show the head/heck/tail sections. Horizontal dashed lines show the 98th percentiles for each group. High values ($\bar{d}_{ij}$ ≥ 98%ile) are labeled with color dots. The upper track shows the location of

completely invariant sites across all sequences (black boxes) and high $\bar{d}_{ij}$ values for each group (color boxes). Numbers shown below are amino acid positions relative to human MYH7 shown for sites with high $\bar{d}_{ij}$ values in at least threewo groups, fourthree groups (*), or all fivefour groups (**). (Art credit for all silhouettes: C. Harvey).