

Georgia Journal of Science

Volume 79 No. 2 *Scholarly Contributions from the Membership and Others*

Article 2

2021

Zebrafish (*Danio rerio*) Hoxb6: An Exploration into the Divergence of Genomic DNA Sequence and Gene Expression Across Teleost Fishes Post-Genome Duplication

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Recommended Citation

Rittgers, Amber Lynn; Le Pabic, Pierre; and Davis, Adam (2021) "Zebrafish (*Danio rerio*) Hoxb6: An Exploration into the Divergence of Genomic DNA Sequence and Gene Expression Across Teleost Fishes Post-Genome Duplication," *Georgia Journal of Science*, Vol. 79, No. 2, Article 2.

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Acknowledgements

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ZEBRAFISH (*DANIO RERIO*) *HOXB6*: AN EXPLORATION INTO THE DIVERGENCE OF GENOMIC DNA SEQUENCE AND GENE EXPRESSION ACROSS TELEOST FISHES POST-GENOME DUPLICATION

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ABSTRACT

Hoxb6 is an evolutionarily conserved developmental regulatory gene that functions, in part, to pattern several organs and organ systems within the embryonic trunk during vertebrate embryogenesis. The *cis*-regulatory circuitry mediating trunk expression in mouse (*Mus musculus*) may be conserved across gnathostome vertebrates, as several other species show similar trunk expression patterns, including chicken (*Gallus gallus*), dogfish shark (*Scyliorhinus canicula*), and several teleost fishes. A whole genome duplication event that occurred in the lineage leading to teleost fishes has generated at least two *Hoxb6* genes, *hoxb6a* and *b6b*. Two teleost fishes of the superorder Acanthopterygii, Japanese medaka (*Oryzias latipes*) and Nile tilapia (*Oreochromis niloticus*), exhibit divergent *Hoxb6* expression patterns from those of non-teleost vertebrates. This includes an anterior expansion of expression for both *hoxb6a* and *b6b* into pharyngeal arch 7, the posterior-most pharyngeal arch that, along with the other posterior pharyngeal arches, gives rise to the pharyngeal jaw apparatus in teleost fishes. While these patterns of expression are observed for both duplicate *Hoxb6* genes in Acanthopterygians, it is uncertain whether this pharyngeal arch expression is shared with other teleost taxa. Here we present the expression patterns of *hoxb6a* and *b6b* in zebrafish (*Danio rerio*), a member of the Ostariophysi superorder. We show that, unlike the strict orthologs from medaka and tilapia, zebrafish *hoxb6a* is expressed in pharyngeal arches 5-7, whereas *hoxb6b* is not expressed in any of the pharyngeal arches. Further, we show through comparative genomic DNA sequence analyses that, although all teleost-specific sequences exhibit moderate conservation with the region functionally tested in mouse, zebrafish *hoxb6a* and *b6b* exhibit little to no conservation in sequence with their strict orthologs of medaka or tilapia outside of this region. Our data suggest that divergence in the *cis*-regulatory circuitry post-genome duplication has generated divergent *hoxb6a* and *b6b* expression patterns among teleost fishes.

Keywords: *Danio rerio*, *Hoxb6a* and *Hoxb6b* gene expression, pharyngeal arches, embryonic development, genome duplication

INTRODUCTION

Hoxb6 is an evolutionarily conserved developmental regulatory gene that functions, in part, to pattern structures within the vertebrate embryonic trunk, including skeletal, central nervous system, digestive, and respiratory structures (Casaca et al. 2016; Kömüves et al. 2000; Rancourt et al. 1994; Sakiyama et al. 2000 and 2001). A whole

genome duplication at the incipient stage of teleost evolution, which occurred 350-220 million years ago (Hoegg et al. 2007; Hurley et al. 2007; Meyer and van de Peer 2005; Mungpakdee et al. 2008; Postlethwait et al. 2004; Santini et al. 2009, Stellwag 1999; Taylor et al. 2001), generated two *Hoxb6* paralogous genes in most teleosts, *hoxb6a* and *b6b* (Amores et al. 1998, 2004; Davis and Stellwag 2010; Kurosawa et al. 2006; Lyon et al. 2013; Prince 2002). The duplication of *Hoxb6* may have facilitated the divergence of gene expression patterns in teleost fishes from those observed for *Hoxb6* in mouse (*Mus musculus*), chicken (*Gallus gallus*), and dogfish shark (*Scyliorhinus canicula*), all of which are restricted to embryonic trunk structures (Becker et al. 1996; Eid et al. 1993; Gaunt and Strachan 1996; Oulion et al. 2011; Sakiyama et al. 2000 and 2001; Schughart et al. 1991). In support, *hoxb6a* and *b6b* expression in representatives of the teleostean superorder Acanthopterygii, Japanese medaka (*Oryzias latipes*) and Nile tilapia (*Oreochromis niloticus*), is expanded anteriorly into the embryonic head to include the posterior-most pharyngeal arch (Davis and Stellwag 2010; Lyon et al. 2013). Under the assumption that the *Hoxb6* ancestral state for gnathostomes is a trunk-restricted expression pattern, the pharyngeal expression of *hoxb6a* and *b6b* would have resulted from cis-regulatory sequence evolution within Acanthopterygii. The evolution of *hoxb6a* and *b6b* pharyngeal expression exemplifies how the diversification of *Hox* gene expression post genome duplication may have promoted the rise in phenotypic adaptations that accompanied the diversification of teleost fishes (Nelson et al. 2016).

The pharyngeal jaw apparatus of teleost fishes is composed of an internal set of jaws that is distinct from the oral jaws and develops from the cranial neural crest cells within the pharyngeal arches (Kimmel et al. 2001; Liem 1973; Nelson et al. 2016; Schaeffer and Rosen 1961). The pharyngeal jaw apparatus played an important role in the success of the teleostean adaptive radiation as its morphological and anatomical diversification expanded the range of ecological niches accessible to teleosts (Liem 1973; Nelson et al. 2016; Schaeffer and Rosen 1961). The variation in morphology and anatomy of the pharyngeal jaw apparatus among teleost fishes may be due, in part, to changes in *Hox* gene expression patterns in the pharyngeal arches. Pharyngeal arch-specific-expression overlaps of *Hox* genes pattern the particular anatomy and morphology of the derivatives of all pharyngeal arches, except for pharyngeal arch 1 (Crump et al. 2006; Minoux et al. 2017; Parker et al. 2018; Santagati et al. 2005). Several studies involving craniofacial development in teleost fishes have documented the pharyngeal arch-specific expression patterns of several *Hox* paralog group 2-5 genes. These include analyses in the ostariophysan zebrafish (Brown et al. 2020; Hogan et al. 2004; Hortopan et al. 2011; Hunter and Prince 2002; Miller et al. 2004; Thorsten et al. 2004) and the acanthopterygian medaka (Davis et al. 2008; Davis and Stellwag 2010), and tilapia (Le Pabic et al. 2007, 2009; Lyon et al. 2013). Interestingly, several of the *Hox* paralog group 2-5 genes of zebrafish were shown to exhibit divergent spatial expression patterns from their orthologous counterparts in medaka, tilapia, or both (Brown et al., 2020), suggesting a lineage-level *Hox* gene expression divergence between superorders Ostariophysi and Acanthopterygii.

Here, we show the expression of zebrafish *hoxb6a* and *b6b* at the pharyngula stage - 48 hours post fertilization (hpf). At this stage, the cranial neural crest cells have migrated to their specific pharyngeal arches (Schilling et al. 1994). *Hox* gene expression

up until late in pharyngeal arch development has been shown to be necessary for the proper patterning of craniofacial skeletal elements (Baltzinger et al. 2005; Gendron-Maguire et al. 1993; Grammatopoulos et al. 2000; Hunter and Prince, 2002; Pasqualetti et al. 2000; Rijli et al. 1993; Santagati et al. 2005). Interestingly, both zebrafish *hoxb6a* and *b6b* show divergent expression from their strict orthologs in medaka and tilapia, such that while both *hoxb6a* and *b6b* are expressed in pharyngeal arch 7 in medaka and tilapia, zebrafish *hoxb6a* is expressed in pharyngeal arches 5, 6, and 7 whereas zebrafish *hoxb6b* expression is not observed in the pharyngeal arches. Further, comparative genomic sequence analyses revealed moderate sequence conservation of the trunk enhancer region among all gnathostomes analyzed. Little to no sequence similarity was detected outside of the trunk regulatory enhancer region between zebrafish *hoxb6a* and *b6b* or their strict orthologs within Acanthopterygii (medaka and tilapia). This lineage-specific divergence in *cis*-regulatory sequence parallels the divergent pharyngeal expression patterns reported here and in previous studies. Finally, we provide an updated and exhaustive account of *Hox* paralog group 2-6 gene expression patterns within the pharyngeal arches of zebrafish, medaka, and tilapia.

MATERIALS & METHODS

Zebrafish *Hoxb6a* and *Hoxb6b* cDNA Cloning

Zebrafish *hoxb6a* and *b6b* partial complimentary DNAs (cDNAs) were generated from reverse transcriptase polymerase chain reaction (RT-PCR) using total RNA isolated from 36 hpf zebrafish embryos as previously described (Westerfield 2000). The primers used for the amplification of *hoxb6a* and *b6b* partial cDNAs were designed based on published zebrafish cDNA sequences (Accession numbers: NM131119 and NM131538; Prince et al. (1998a, b, c)) to amplify a 563 bp and 655 bp fragment of each transcript, respectively (ZebB6aFor: 5'- ACTTTCCCAGAGACTCTG-3' and ZebB6aRev: 5'- TTCGCCGGTTTTGGAACC-3'; ZebB6bFor: 5'- CTCAACTTTTCCCGTGTC-3'; ZebB6bRev: 5'- TTATCCAGCCTTTCACC-3'). PCRs were performed in 50 μ L volumes containing 25 μ L OneTaq 2X Master Mix with Standard Buffer (New England Biolabs, Ipswich, MA), 5 μ L of 3 pmol/ μ L for each forward and reverse primer, 4 μ L cDNA, and 11 μ L nuclease-free molecular grade water (ThermoFisher Scientific, Waltham, MA). PCR conditions were as follows: 1 min at 94 °C, 34 cycles of 45 sec at 94 °C, 30 sec at 54 °C, and 45 sec at 72 °C, and 10 min at 72 °C. PCR products were subcloned in TOPO TA dual promoter pCR II vectors (Invitrogen, Carlsbad, CA) and cloned into One Shot Top10 chemically competent *E. coli* (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Plasmid DNAs were isolated using Plasmid Miniprep Kit (Sigma-Aldrich, St. Louis, MO). Confirmation and orientation of PCR products corresponding to inserts from plasmid cDNA clones were determined by restriction endonuclease digestion.

Whole-Mount *In Situ* Hybridization

Forty-eight hpf zebrafish embryos were paraformaldehyde-fixed, dechorionated, and methanol according to protocol #A1617-006 approved by the University of North Carolina, Wilmington Institutional Animal Care and Use Committee. At this developmental stage, the pharyngeal arches are well segmented and easily

distinguishable. An earlier time point (20 somite-stage) has previously been reported (Grandel et al. 2002; Prince et al. 1998a). Whole-mount *in situ* hybridization was performed following the standard operating procedure published in Davis et al. (2019). Production and purification of digoxigenin (DIG)-labeled sense and antisense riboprobes and development of DIG-labeled signal were performed according to Scemama et al. (2006). Embryos were photographed using a Motic BA210E compound light microscope and a Moticam X3 digital camera (Motic, Feasterville, PA). Images were processed using Adobe Photoshop.

Comparative Genomic DNA Sequence Analysis

The *Hoxb6*, *hoxb6a*, and *hoxb6b* genes, introns, and upstream intergenic regions were analyzed from organisms with known expression patterns using mVISTA (<http://http://genome.lbl.gov/vista/index.shtml>) (Frazer et al 2004; Mayor et al. 2000). These included mouse (Accession number: CM000219), chicken (NC006114), dogfish shark (FQ032659), zebrafish (AL645782 and NC007123), medaka (AB232920 and AB232921), and tilapia (GCA_001858045.3 for both *hoxb6a* and *b6b*). The genomic DNA regions included in the analysis encompassed *Hoxb6* of mouse, chicken, and dogfish shark and *hoxb6a* and *b6b* of zebrafish, medaka, and tilapia and roughly 5000-10,000 bp upstream, depending on the size of the species-specific upstream intergenic region. The Shuffle-LAGAN option in mVISTA, which detects rearrangements and inversions, was used for sequence alignment. The following parameters were selected in the presentation of results: window 100 bp, minimum conservation width of 100 bp, and conservation identity of 70%. Reference sequences used for presentation of results included mouse *Hoxb6*, zebrafish *hoxb6a*, and zebrafish *hoxb6b*. Mouse *Hoxb6* was used as a reference sequence to define the upstream regulatory region and to show the degree of conservation of this region in the teleost genomes examined in this study. Zebrafish *hoxb6a* and *hoxb6b* were used as reference sequences to see if there was any sequence conservation outside of the upstream regulatory region between these paralogs and with their strict orthologs, *hoxb6a* and *hoxb6b* of medaka and tilapia.

RESULTS

Zebrafish *Hoxb6a* Expression Pattern

We observed zebrafish *hoxb6a* expression in pharyngeal arches 5, 6 and 7 at 48 hpf (Figure 1A and B). This expression pattern was not observed at the earlier 20 somite stage, in which *hoxb6a* was previously reported with an anterior limit of expression at somite two (Grandel et al. 2002; Prince et al. 1998a). This pattern of expression is divergent from its strict orthologs in both medaka and tilapia, both of which are expressed in pharyngeal arch 7 alone (Davis and Stellwag 2010; Lyon et al. 2013). However, the timing of expression was shown to be similar to medaka *hoxb6a*, for which pharyngeal expression occurred only during the late pharyngula stage (Davis and Stellwag 2010).

Zebrafish *Hoxb6b* Expression Pattern

We observed zebrafish *hoxb6b* expression to be restricted to the embryonic trunk at 48 hpf (Figure 1C). A similar expression pattern that is posterior to the pharyngeal

arches was previously reported at the earlier 20 somite stage (Grandel et al. 2002; Prince et al. 1998a). The lack of zebrafish *hoxb6b* expression in the pharyngeal arches is divergent from *hoxb6b* in both medaka and tilapia, which are both expressed in pharyngeal arch 7 (Davis and Stellwag 2010; Lyon et al. 2013).

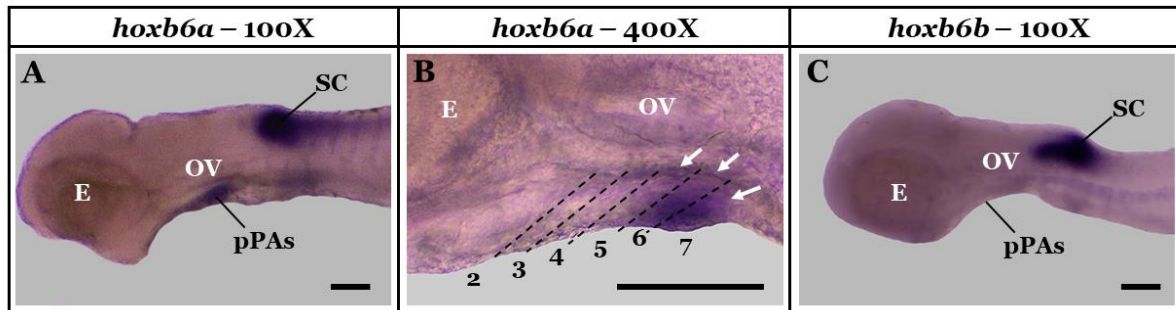
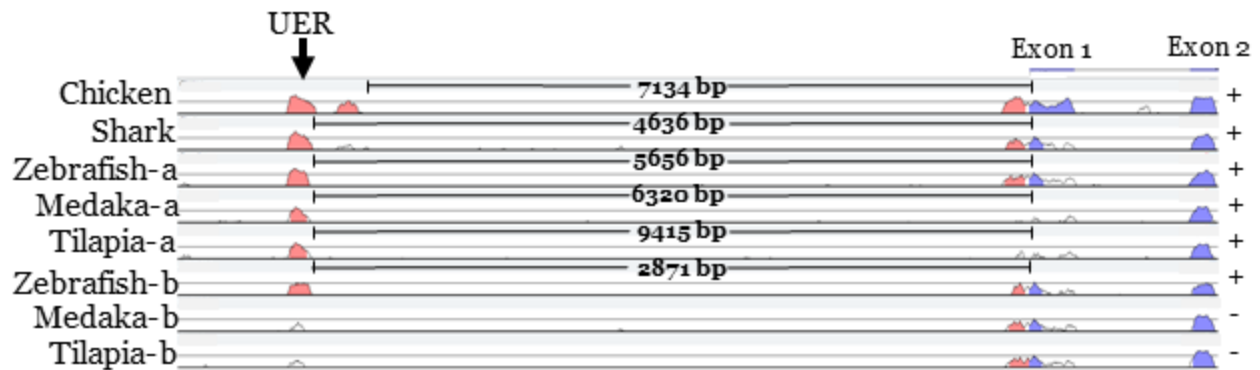


Figure 1. Whole mount *in situ* hybridization analysis of zebrafish *hoxb6a* at 100X (A) and 400X magnification (B) and *hoxb6b* at 100X magnification in 48 hpf embryos. All embryos were mounted with their anterior sides facing left and lateral sides facing the reader. Numbers below the ventral side of the embryo in panel B correspond to the pharyngeal arches. Expression of *hoxb6a* and *b6b* within the spinal cord represents a positive control for the detection of pharyngeal arch expression. E, eye; OV, otic vesicle; pPAs, posterior pharyngeal arches; SC, spinal cord. Scale bars equal 0.1 mm.

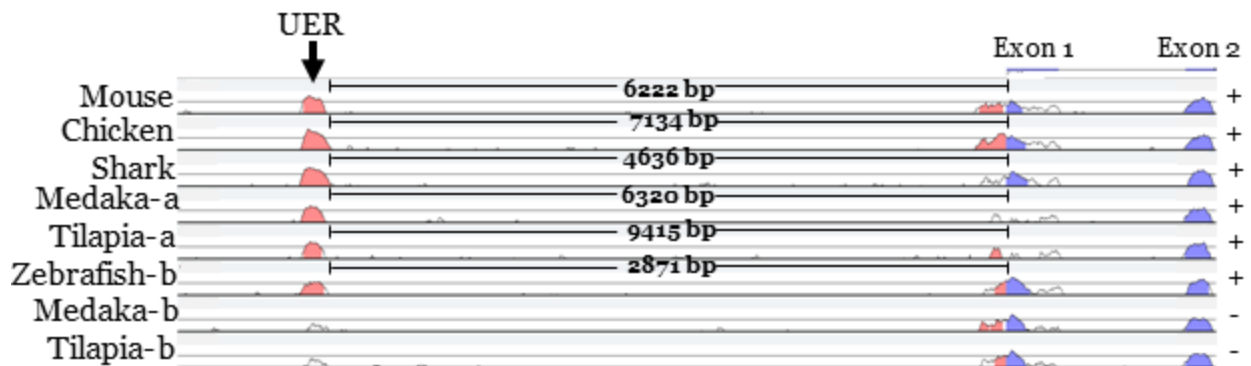
Comparative Genomic Sequence Analysis

A large upstream intergenic region containing several enhancer elements that direct *Hoxb6* gene expression in several trunk tissues during embryonic development has been identified in mouse (Becker et al. 1996; Eid et al. 1993; Schughart et al. 1991; Sharpe et al. 1998). We observed that outside of this region, there was little to no sequence conservation between mouse and any of the teleost fishes and, surprisingly, little to no conservation between zebrafish *hoxb6a* and *b6b* genes and their strict orthologs in medaka and tilapia (Figure 2A-E). Further, we observed varying lengths of upstream intergenic sequence between the upstream enhancer region and the ATG start site for all species assayed. These lineage-specific divergences in sequence may parallel the divergent pharyngeal arch expression patterns reported here and in previous studies.

Reference sequence: *Mouse Hoxb6*



Reference sequence: *Zebrafish hoxb6a*



Reference sequence: *Zebrafish hoxb6b*

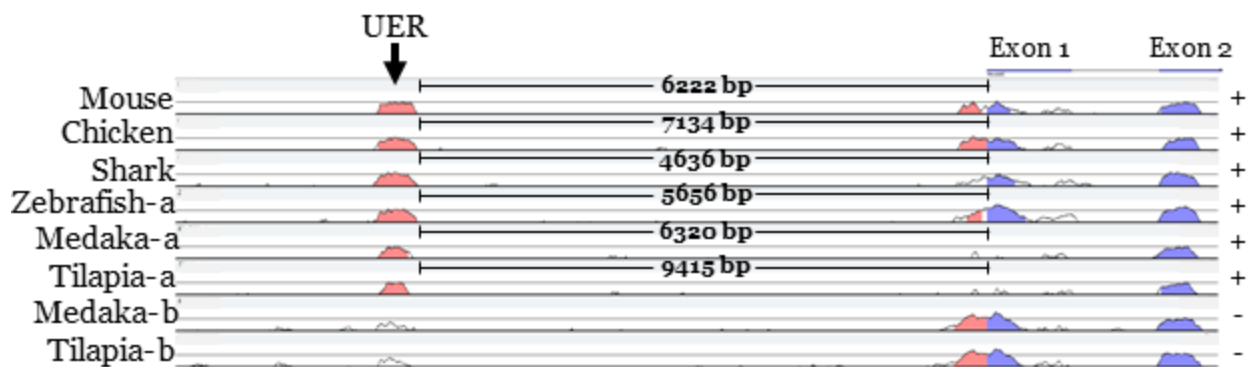


Figure 2. mVista sequence alignment plot for *Hoxb6* of mouse, chick, and shark and *hoxb6a* and *b6b* of zebrafish, medaka, and tilapia. The plot encompasses the exons, introns and ~5,000-10,000 bp of upstream intergenic DNA. Peaks shown within each frame represent the levels of sequence similarity in a 100 bp window. Blue-shaded peaks and red-shaded peaks correspond to exons and conserved noncoding DNA sequences, respectively, that are at or above 70% conservation identity with respect to the reference sequence. White, or uncolored, peaks correspond to coding or noncoding sequences that are below 70% conservation identity with respect to the reference sequence. All peaks correspond to DNA sequences compared to the reference sequences, which include mouse *Hoxb6*, zebrafish *hoxb6a*, and zebrafish *hoxb6b*. Arrows denote the orthologous conserved upstream enhancer region functionally tested in mouse (Becker et al. 1996; Eid et al. 1993; Schughart et al. 1991; Sharpe et al. 1998). Variation in position

of the upstream enhancer region between plots is due to differential lengths in intergenic sequences analyzed. Lengths of intergenic sequences between the upstream enhancer region and the ATG start site of the *Hoxb6* genes are shown in the figure. Pluses (+) correspond to the presence of a conserved upstream enhancer region that shares 70% conservation identity with the reference sequences. Minuses (-) correspond to the lack of a conserved upstream enhancer region, or a region that has less than 70% conservation identity with the reference sequences. Teleost *hoxb6a* sequences correspond to zebrafish-a, medaka-a, and tilapia-a. Teleost *hoxb6b* sequences correspond to zebrafish-b, medaka-b, and tilapia-b. UER, upstream enhancer region.

DISCUSSION

Our expression pattern and comparative genomic analyses of zebrafish *hoxb6a* and *b6b* show divergence from their strict orthologs in medaka and tilapia. For instance, while medaka and tilapia *hoxb6a* and *b6b* are both expressed in pharyngeal arch7 alone (Davis and Stellwag 2010; Lyon et al., 2013), zebrafish *hoxb6a* is expressed in pharyngeal arches 5-7 whereas *hoxb6b* is not expressed within the pharyngeal arches (see Figure 3). Interestingly, these divergent expression patterns coincide with divergent genomic sequences upstream of *hoxb6a* and *b6b*. While all teleost *hoxb6a* and *b6b*-specific sequences show varying degrees of conservation with the upstream regulatory region of mouse *Hoxb6*, a region that has been shown to direct *Hoxb6* expression in the embryonic trunk (Becker et al. 1996; Eid et al. 1993; Schughart et al. 1991; Sharpe et al. 1998), little to no conservation was detected outside of this region between zebrafish *hoxb6a* or *b6b* and their strict orthologs in medaka and tilapia. However, it must be stressed that regions located outside the genomic interval analyzed in this study may be involved in directing *hoxb6a* and *b6b* pharyngeal arch expression. Many *Hox* gene expression patterns are regulated by *cis*-regulatory elements located upstream, downstream, within introns, and even within coding regions (Tümpel et al., 2009). In addition, conserved *cis*-regulatory sequences do not necessarily have conserved functions: a highly conserved enhancer region upstream of gnathostome *Hoxa2* paralogs drives expression in rhombomere 4 in medaka but neither in mouse nor chicken (Davis et al. 2016; Maconochie 1999 and 2001; Tümpel et al., 2002 and 2009). Functional tests of the conserved upstream enhancer region of *hoxb6a* and *b6b* of teleosts may help to shed light on whether this region has diverged in function between ray-finned fishes (Actinopterygii) and tetrapods (Sarcopterygii), and also within teleosts between acanthopterygians and ostariophysans.

The shared pharyngeal arch 7 expression of zebrafish *hoxb6a* with medaka and tilapia *hoxb6a* and *b6b* suggests that pharyngeal arch 7 expression was part of the teleost *hoxb6* ancestral expression pattern pre-genome duplication. Such a pattern would suggest evolutionary constraint on the *cis*-regulatory elements that direct pharyngeal expression of *hoxb6a* and *b6b* in acanthopterygians. By contrast, this pattern would suggest a loss of expression of zebrafish *hoxb6b* in this arch, but a gain of expression of zebrafish *hoxb6a* in pharyngeal arches 5 and 6. Based on this scenario, it is also tantalizing to suggest that the gain of *hoxb6a* expression within these arches in zebrafish may have compensated for a loss of pharyngeal expression of other *Hox* genes. In support, while *hoxd4a* of both tilapia and medaka are expressed in pharyngeal arches 4-7, the zebrafish ortholog is constrained to just pharyngeal arches 4 and 5 (Brown et al.

2020; Davis and Stellwag 2010; Le Pabic et al. 2009; Lyon et al. 2013). Further, while *hoxd4b* is expressed in pharyngeal arches 5-7 in medaka and tilapia, this gene was lost to nonfunctionalization in the lineage leading to zebrafish (Amores et al. 1998; Brown et al. 2020; Davis et al. 2010; Lyon et al. 2013; Prince et al. 2002). Several genetic analyses have shown that *Hox* genes are involved in multiple auto- and cross-regulatory interactions within the developing pharyngeal arches, and that these interactions are necessary for the proper patterning of the craniofacial skeletal elements (Hunter and Prince 2002; Le Pabic et al. 2010; Minoux et al. 2007; Parker et al. 2018). Such interactions suggest a selective constraint on genetic regulatory networks involving multiple *Hox* genes within the pharyngeal arches. Obtaining *hoxb6a* and *b6b* expression patterns from other teleost fishes such as the three-spine stickleback (*Gasterosteus aculeatus*) and Atlantic killifish (*Fundulus heteroclitus*), may help resolve the ancestral state of *hoxb6* for osteichthyans. Interestingly, dogfish shark *Hoxb6* was shown to not be expressed in the pharyngeal arches (Oulion et al., 2011). Providing that other chondrichthyan species do not exhibit pharyngeal arch-specific *Hoxb6* expression, these results are suggestive of *Hoxb6* expression within the arches as a derived characteristic of ray-finned fishes. Analysis of *Hoxb6* gene expression in the pharyngeal arches of basal, non-teleost osteichthyan lineages, such as *Polypteriformes*, would also help resolve the osteichthyan ancestral state.

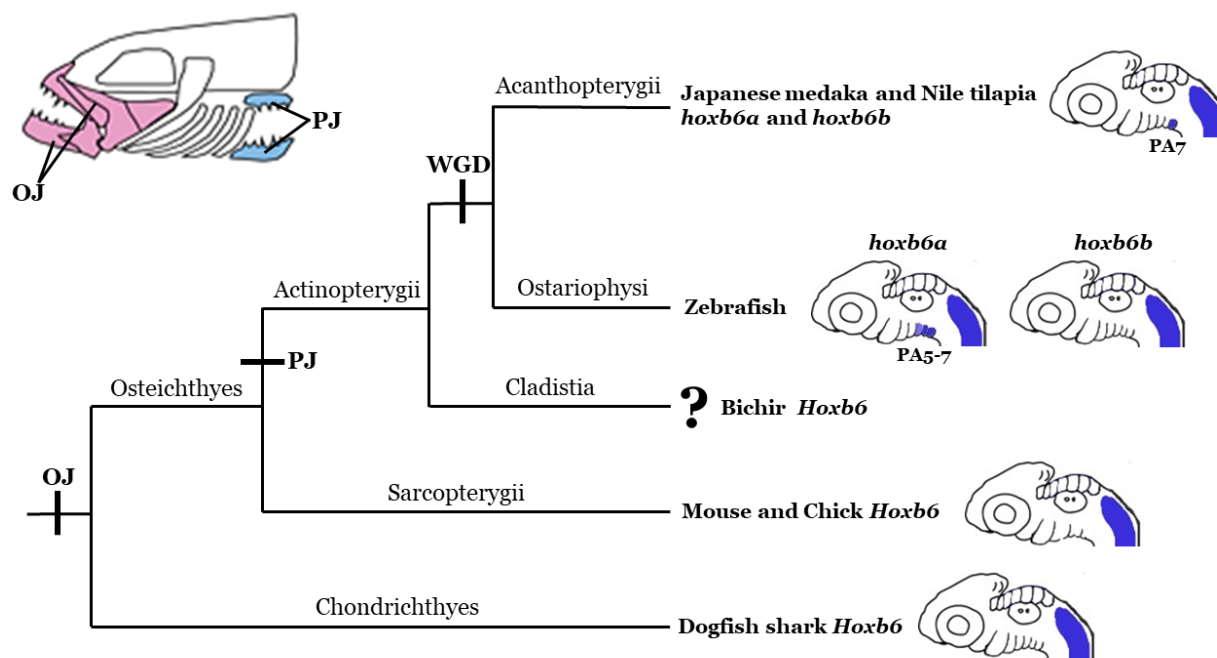


Figure 3. *Hoxb6* gene complement and anterior expression pattern evolution in the Gnathostomata. Drawings represent left-facing pharyngula-stage embryonic heads. Blue coloration represents embryonic gene expression patterns. The skull with jaws in the upper left corner of the figure shows the oral and pharyngeal jaws within the Actinopterygii. Phylogeny based on Betancur et al. (2017). OJ, oral jaws; PA, pharyngeal arch; PJ, pharyngeal jaws; WGD, whole genome duplication.

In addition to their divergent *hoxb6a* and *b6b* pharyngeal expression patterns, zebrafish, medaka, and tilapia each exhibit divergent *Hox* paralog group 2-5 gene expression patterns in the pharyngeal arches (Figure 4). This great diversity of expression in teleost pharyngeal arches resulted from the differential loss of particular duplicates in particular lineages post-genome duplication as well as lineage-specific cis-regulatory evolution. For instance, while medaka has lost *hoxa2b*, zebrafish has lost *hoxa2a*, *b3b*, and *d4b* (Amores et al. 1998; Davis et al. 2008). Divergence in both cis-regulatory machinery and gene content may have allowed for divergent genetic regulatory networks and thus variation in the molecular patterning of the pharyngeal arch bony derivatives among teleosts. For instance, divergent morphological features in the pharyngeal jaw apparatus include fused lower jaw bones and a diarthrotic articulation between elements of the upper jaw with the ventral side of the neurocranium in tilapia (Liem 1973; Stiassny and Jensen 1987), reduced size of the second and third epibranchials, expanded articular surface of the fourth epibranchial, and the presence of large ventral flanges on the fifth ceratobranchial in medaka (Langille and Hall 1987; Parenti 1987; Rosen and Parenti 1981), and enlargement of the fifth ceratobranchial with teeth ankylosed to the bone and the absence of the first pharyngobranchial in zebrafish (Nelson et al., 2016). Such profound differences in phenotype, and ultimately life histories, likely resulted from evolution in the genetic regulatory networks which regulate the morphogenesis of these lineage-specific characteristics during embryogenesis.

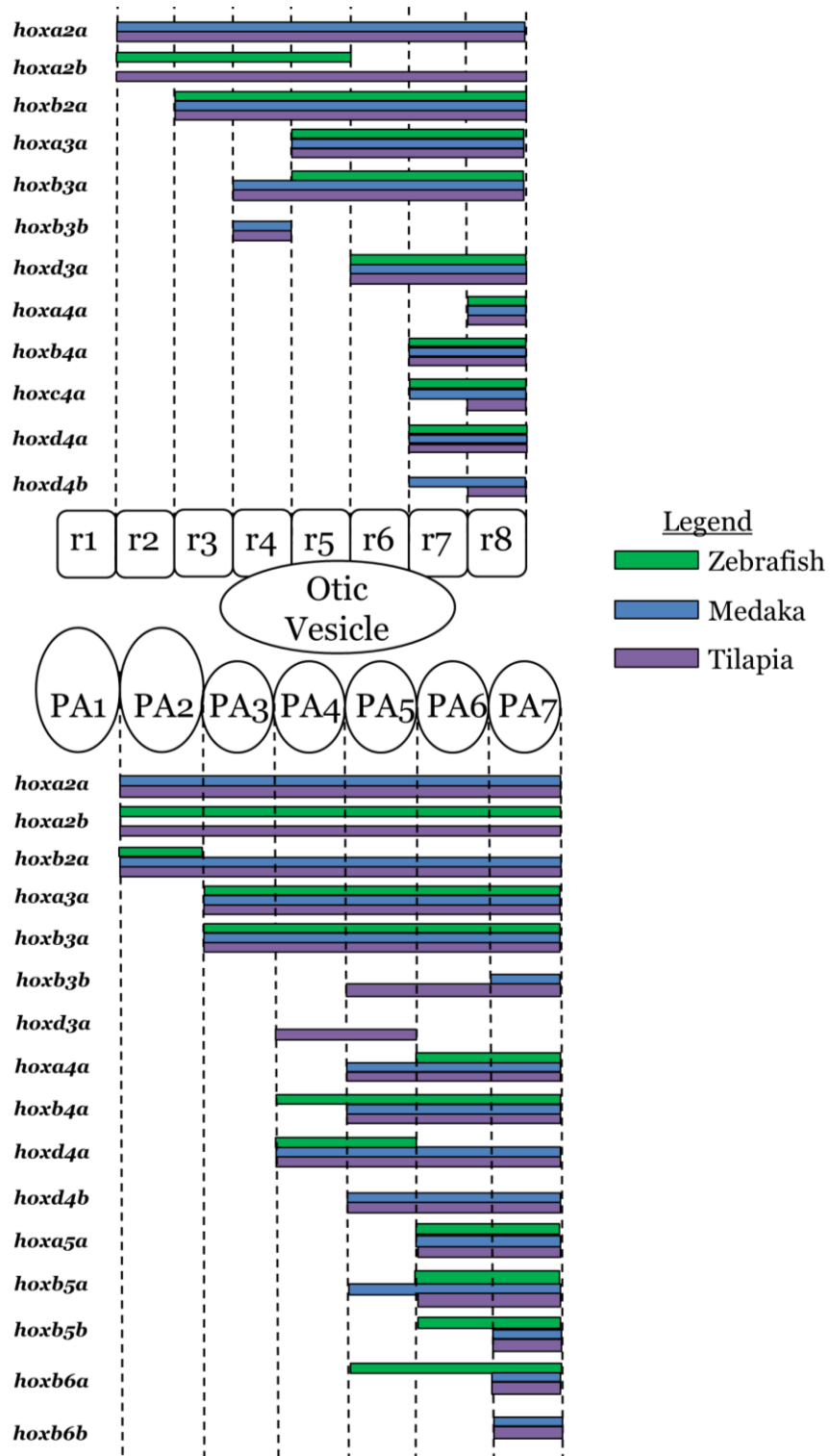


Figure 4. Comparative combinatorial code of *Hox* paralog group 2-6- gene expression in the hindbrain and pharyngeal arches during post-migratory cranial neural crest cell stages in zebrafish (green bars), medaka (blue bars), and tilapia (purple bars). All rhombomere and most pharyngeal arch expression patterns are derived from this paper and from the literature (Brown et al. 2020; Davis et al. 2008; Davis and Stellwag 2010; Hogan et al. 2004; Hortopan et al. 2011;

Hunter and Prince 2002; Le Pabic et al. 2007 and 2009; Lyon et al. 2013; Miller et al. 2004; Thorsten et al. 2004). PA, pharyngeal arch; r, rhombomere.

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