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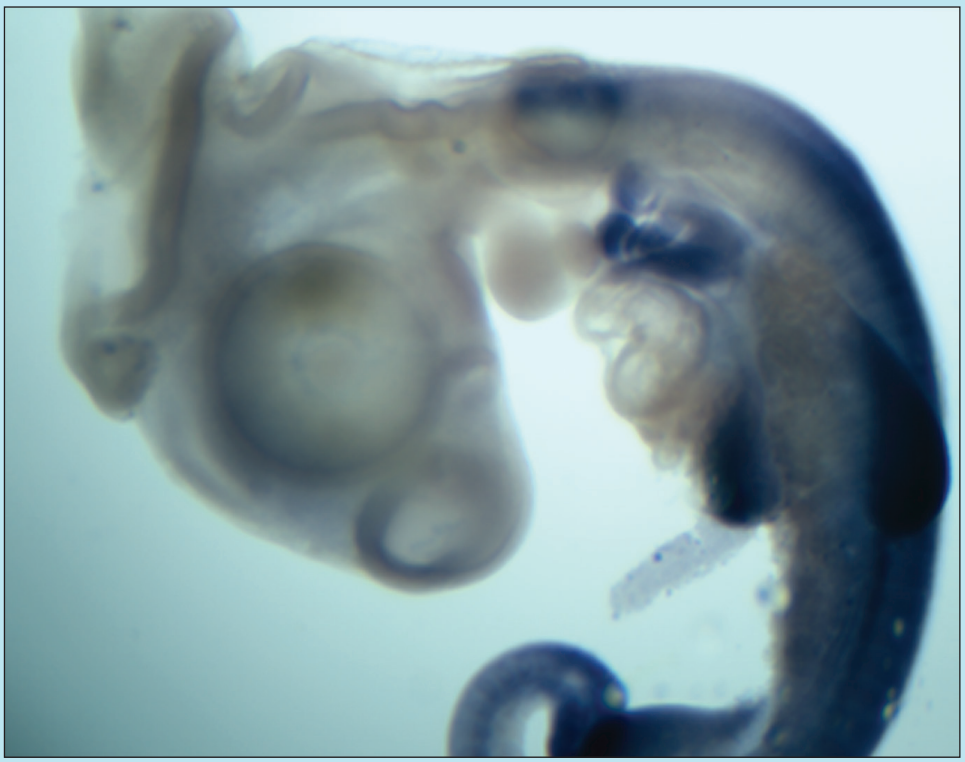

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Cover Photograph: *Hoxa3* gene expression in the Brown anole lizard (*Anolis sagrei*) embryo. Photo taken by Adam Davis at University of North Georgia.

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An Analysis of *Hoxa3* Gene Expression and Regulation across Gnathostomes

Benjamin S. Wortman[1], Shafiya M.S. Khan[2], Amber L. Rittgers[3], John McGinnis[1], Michael A. White[1], and Adam Davis[4*]

Abstract - *Hoxa3* is an evolutionarily conserved developmental regulatory gene that is expressed within the posterior rhombomeres (r) and pharyngeal arches (PA). Previous molecular genetic studies have shown that *Hoxa3* in tetrapods and smaller spotted catsharks, as well as *hoxa3a*, *hoxa3a*α and *hoxa3a*β in teleost fishes, show a conserved anterior limit of expression in r5 and PA3. Further, a conserved enhancer region, termed the r5/r6 region, upstream of *Hoxa3* in the mouse and chicken was shown to direct *Hoxa3* expression at these anterior limits. In this study, we show that *Hoxa3* of *Anolis sagrei* (brown anole) and *hoxa3a* of *Gasterosteus aculeatus aculeatus* (three-spine stickleback) exhibit conserved anterior limits of expression. However, our bioinformatics analyses show that, while *Hoxa3* in brown anoles and other tetrapods, the coelacanth, the smaller spotted catshark, and the spotted gar, exhibit conservation with the functional sequences tested in mice and chickens, these sequences have diverged in teleost fishes. This divergence may be due to the whole-genome duplication that occurred in the lineage leading to teleosts.

Introduction

The anterior-posterior (A-P) axis during vertebrate embryonic development is segmented into transient compartments that ultimately differentiate and give rise to morphological structures that make up the vertebrate body plan (McGinnis and Krumlauf 1992). The compartments of the head region include the rhombomeres (r) of the hindbrain, which give rise to the cranial nerves, and the pharyngeal arches (PAs), which give rise to several craniofacial bones, including the jaws and inner ear bones (Graham 2003, Krumlauf and Wilkinson 2021). The identities of the rhombomeres and PAs are patterned, in part, by *Hox* genes, which encode evolutionarily conserved transcription factors that regulate the expression of downstream genes involved in morphogenesis (Krumlauf and Wilkinson 2021, Kuratain 2004, Minoux et al. 2009, Tümpel et al. 2009). *Hox* genes are arranged in clusters and exhibit spatial and temporal collinearity, such that more 3'-located genes within a cluster are expressed earlier and more anteriorly during development (Ferrier et al. 2000, Holland and Garcia-Fernandez 1996, Powers and Amemiya 2004). Further, several, if not most, *Hox* genes exhibit nested expression patterns along the A-P axis, which allows them to be involved in both auto- and cross-regulatory mechanisms of expression (Tümpel et al. 2009).

Hoxa3, one of the earliest and anterior-most expressing *Hox* genes, shows a conserved expression pattern across all vertebrates analyzed to date, with anterior limits of expression occurring in r5 and PA3. This pattern has been observed in several highly evolutionarily divergent tetrapod vertebrates, including *Mus musculus* Linnaeus (Mouse), *Gallus gallus* Linnaeus (Chicken), *Pantherophis guttatus* Linnaeus (Corn Snake), and *Xenopus laevis*

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Daudin (African Clawed Frog), as well as the chondrichthyan *Scyliorhinus canicula* Linnaeus (Smaller Spotted Catshark) (Chojnowski et al. 2016; Guidato et al. 2003; Kameda 2009; Lee et al. 2013; Manzanares et al. 1999, 2001; McNulty et al. 2005; Oulion et al. 2011; Watari-Goshima and Chisaka 2011; Woltering et al. 2009). Interestingly, *hoxa3a* of several teleost fishes, including *Danio rerio* Hamilston (Zebrafish), *Oryzias latipes* Temminck and Schlegel (Japanese Medaka), *Oreochromis niloticus* Linnaeus (Nile Tilapia), and *Takifugu rubripes* Temmink and Schlegel (Japanese Pufferfish) and *hoxa3a*α and *hoxa3a*β of *Salmo salar* (Atlantic Salmon) show a similar expression pattern to *Hoxa3* of tetrapods and the smaller spotted catshark (Amores et al. 2004, Davis and Stellwag 2010, Hogan et al. 2004, Le Pabic et al. 2009, Mungpakdee et al. 2008b). Several lines of genetic and phylogenetic evidence have shown that the lineage leading to teleost fishes underwent a whole genome duplication (Amores et al. 1998, 2004; Hoegg et al. 2007; Kurosawa et al. 2006; Moghadam et al. 2005; Prince 2002; Stellwag 1999; Thomas-Chollier et al. 2007), thus resulting in two *Hoxa3* genes, *hoxa3α* and *hoxa3β*. However, post-genome duplication independent gene loss in this lineage has resulted in the retention of *hoxa3a* alone across most teleost fishes (Amores et al. 1998, 2004; Davis and Stellwag 2010; Hoegg et al. 2007; Hogan et al. 2004; Le Pabic et al. 2009; Kurosawa et al. 2006; Moghadam et al. 2005; Prince 2002; Stellwag 1999; Thomas-Chollier et al. 2007). A subsequent genome duplication in the lineage leading to Atlantic salmon has resulted in two *hoxa3a* genes, *hoxa3a*α and *hoxa3a*β (Mungpakdee et al. 2008a, b).

The conservation of tetrapod and shark *Hoxa3* and teleost *hoxa3a*, *hoxa3a*α, and *hoxa3a*β expression patterns in the rhombomeres and PAs is suggestive of conserved *cis*-regulatory elements (CREs), or small genomic DNA sequences that bind transcription factors and direct when and where these genes are expressed. Indeed, this was tested and observed with both bioinformatics-based comparisons between mice, chickens, sharks, and humans and functional reporter gene assays within the mouse and chicken model systems (Manzanares et al. 1999, 2001). A conserved enhancer region was shown to be located upstream of mouse and chicken *Hoxa3* and includes CREs specific for Prep/Meis, Kreisler, and Hox/Pbx transcription factors (Manzanares et al. 1999, 2001). This region, known as the r5/r6 enhancer, was shown to drive *Hoxa3* expression in r5 and 6 as well as the neural crest migrating from these rhombomeres and populating the posterior PAs (Manzanares et al. 1999, 2001). However, it is unknown if this enhancer region is conserved in gnathostomes beyond mice, humans, chickens, and sharks, including teleost fishes.

Here, we show the expression patterns of *Anolis sagrei* Cocteau (Brown Anole Lizard) *Hoxa3* and *Gasterosteus aculeatus aculeatus* Linnaeus (Three-spine Stickleback) *hoxa3a* during embryonic development. We show that both brown anole *Hoxa3* and stickleback *hoxa3a* exhibit anterior limits of expression at r5 and PA3, which is conserved with all other gnathostomes with characterized *Hoxa3*, *hoxa3a*, *hoxa3a*α and *hoxa3a*β expression patterns. However, we observed divergence within the r5/r6 enhancer region. Specifically, we show that the r5/r6 enhancer region is conserved among most evolutionarily divergent gnathostomes, including tetrapods, *Latimeria chalumnae* Smith (Coelacanth), an extant relative of tetrapods within the Sarcopterygii (Pough et al. 2019), the smaller spotted catshark, and the *Lepisosteus oculatus* Winchell (Spotted Gar), an extant relative of teleost fishes in the Actinopterygii (ray-finned fishes) (Pough et al. 2019). However, with respect to teleost fishes, we found divergence with the genomic structure of this region. Specifically, we show that teleost fishes lack the r5/r6 enhancer region. These data show that while divergence has occurred within the genomic regulatory circuitry, the conserved expression patterns have been retained among gnathostomes. Further, they suggest that relaxed

selective constraint has occurred with the r5/r6 enhancer region for teleost *hoxa3a*, *hoxa3a*α and *hoxa3a*β. This may be due to the whole-genome duplication event that occurred within the lineage leading to teleost fishes.

Materials and Methods

Brown anole *Hoxa3* and three-spine stickleback *hoxa3a* cloning

Brown anole lizard embryos were obtained from the Genetics Department of University of Georgia (kind gift from Dr. Doug Menke). RNA was extracted from stage 2 and 3 lizard embryos and stage 19-21 stickleback embryos using TRIzol (Invitrogen, Carlsbad, CA) (Sanger and Losos 2008, Swarup 1958). All analyses involving stickleback embryos were performed under IACUC #A2021 07-031-A4. Lizard and stickleback complimentary DNAs (cDNAs) were generated from RNAs using the SuperScript III One-Step RT-PCR System following the manufacturer's instructions (Invitrogen, Carlsbad, CA). The primers used for amplification of the entire coding sequence of brown anole lizard *Hoxa3* cDNA were designed based on the sequenced genome of *Anolis sagrei* (kind gift from Dr. Doug Menke) (LizA3For: 5'-ATGCAAAAAGCGACCTATTACG-3'; LizA3Rev: 5'-TTACAGATGGGTCAATTTGGGG-3'). The primers for stickleback *hoxa3a* cDNA were designed based on the Genbank accession (Accession #: XM_040188607.1) (StickA3aFor: 5'-ATGCAAAAGGCAACCTACTAC-3'; StickA3aRev: 5'-CTACAGATGCGTCAGTTTGG-3'). PCR was performed in a 50 μL volume containing 25 μL of One Taq 2X Master mix with Standard Buffer (New England Biolabs, Ipswich, MA), 5 μL of 3 pmol/μL of both forward and reverse primers, 1 μL cDNA, and 14 μL 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 55 °C, and 45 sec at 72 °C, and 10 min at 72 °C. PCR products were subcloned into PCRII TOPO TA plasmid 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 from *E. coli* using Plasmid DNA Miniprep kits (Sigma-Aldrich, St. Louis, MO) following the manufacturer's instructions. Confirmation and orientation of PCR product corresponding to the insert from the plasmid cDNA clone was determined by enzyme restriction digestion analysis (New England Biolabs, Ipswich, MA) and DNA sequencing (GENEWIZ, South Plainfield, NJ).

Whole mount *in situ* hybridization

Lizard embryos were extracted from eggs using microdissection forceps and scissors (ThermoFisher Scientific, Waltham, MA) on a Motic SMZ-171 Stereo Zoom Microscope (Motic, Feasterville, PA) and developmentally staged according to Sanger et al. (2008). Stage 1-3 lizard embryos were fixed overnight at 4 °C in 4% paraformaldehyde (PFA) (ThermoFisher.Scientific, Waltham, MA). Stage 19-21 stickleback embryos were staged according to Swarup (1958), fixed overnight in 4% PFA at 4 °C, and dechorionated using microdissection forceps on a Motic SMZ-171 Stereo Zoom Microscope. All embryos were then dehydrated in a graded series of methanol (ThermoFisher Scientific, Waltham, MA) with 1X phosphate buffered saline with 0.1% Tween 20 (PBST) (ThermoFisher Scientific, Waltham, MA), and stored in 100% methanol at -20 °C until use.

Whole mount *in situ* hybridization (WISH) assays were performed in 1.7 mL microcentrifuge tubes (ThermoFisher Scientific, Waltham, MA) for stickleback embryos and 4 mL glass vials (ThermoFisher Scientific, Waltham, MA) for lizard embryos. All reactions

utilized 2-5 embryos. Two reactions were performed per each embryo type (brown anole and three-spine stickleback). All steps outlined in the standard operating procedure (SOP) published by Davis et al. (2019) were used with modifications. All volumes used in the steps of the SOP were used in the analysis for stickleback embryos but doubled for lizard embryos based on their size. Proteinase K (ThermoFisher Scientific, Waltham, MA) were performed at room temperature for 20 min for stickleback embryos and 30 min for lizard embryos.

Production and purification of sense and antisense digoxigenin-labeled riboprobes were performed using a SP6/T7 Transcription Kit (Roche, Indianapolis, IN) and a QIAquick PCR Purification Kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. All embryos were mounted on microscope slides using a Motic SMZ-171 Stereo Zoom Microscope (Motic, Feasterville, PA) and photographed using an Amscope B490 compound microscope and associated 10 megapixel camera (Amscope, Irvine, CA). Images were further processed using Adobe Photoshop (Adobe, San Jose, CA). Several morphological landmarks were used to determine the expression pattern of *Hoxa3* and *hoxa3a*, including PAs, rhombomeres, otic vesicle (or developing ear), and the somites.

Comparative genomic DNA sequence analysis

Hoxa3, *hoxa3a*, *hoxa3a*α, and *hoxa3a*β bioinformatics analyses using mVISTA http://genome.lbl.gov/vista/index.shtml) and Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) were used to examine the functionally tested r5/r6 enhancer region for *Hoxa3* of the mouse and chicken with other gnathostomes (Frazer et al. 2004, Madeira et al. 2019). Sequences spanning from the *Hoxa4* start codon to the *Hoxa3* stop codon (or *hoxa4a* to *hoxa3a*, *hoxa4a*α to *hoxa3a*α, and *hoxa4a*β to *hoxa3a*β for teleost fishes) were retrieved from Genbank and Ensembl. These included sequences from species with known *Hoxa3*, *hoxa3a*, *hoxa3a*α, and *hoxa3a*β expression patterns, including mice (Accession #: NC_000072.7), chickens (NC_052574.1), corn snakes (NW_023010903.1), clawed frogs (NC_030682.2), zebrafish (NC_007130.7), Japanese medaka (AB232918.1), Nile tilapia (GCA_001858045.3), Japanese pufferfish (DQ481663.1), Atlantic salmon (NW_012337988 and NW_012341469), and smaller spotted catsharks (FQ032658.1) and as well as sequences for the species tested in this study: three-spine sticklebacks (NC_053221.1) and brown anole lizards (genome of *A. sagrei* kindly supplied by Dr. Doug Menke). We also included the *Hoxa4-Hoxa3* genomic sequences from humans (NT_086366.1), coelacanths (NW_005819160.1), a sarcopterygian species that shares a most recent common ancestor with tetrapods, and spotted gar (NW_006269981.1), an actinopterygian species that shares a most recent common ancestor with the teleost fishes but resides outside of the whole genome duplication event that occurred in the lineage leading to teleost fishes (Pough et al. 2019). The Shuffle-LAGAN option in mVISTA was used for detecting rearrangements and inversions for the genomic DNA sequence alignment. The parameters used for sequence analysis in mVISTA were as follows: window 100 bp, minimum conservation width of 100 bp, and conservation identity of 70%. The mouse *Hoxa4-Hoxa3* genomic sequence was used as the reference sequence in the mVISTA analysis since the CREs of the r5/r6 enhancer region were characterized in this system (Manzanares et al. 2001). Genomic DNA sequence regions of other species that were observed to be conserved with the r5/r6 region upstream of mouse *Hoxa3* were further aligned and analyzed using Clustal Omega. All default parameters were used for the Clustal Omega sequence alignments. Other genomic regions outside of the r5/r6 enhancer region that showed conservation with the mouse *Hoxa4-Hoxa3* intergenic region were not analyzed, as these regions were not shown to direct *Hoxa3* expression within r5/r6 (Manzanares et al. 1999).

Results

Brown anole *Hoxa3* and three-spine stickleback *hoxa3a* expression patterns

We observed both brown anole *Hoxa3* and stickleback *hoxa3a* to show anterior limits of expression in r5 and PA3 (Fig. 1A and 1B). Further, both genes were expressed in the neural tube posterior to the hindbrain and the remaining posterior PAs, including PA4 for the brown anole and PA4-6 for the stickleback. All experimental embryos for both species (4 for the brown anole and 10 for the three-spine stickleback) showed these results. These patterns of expression were shown to be similar to *Hoxa3* in other tetrapods and the smaller spotted catshark and *hoxa3a*, *hoxa3a*α and *hoxa3a*β in other teleost fishes (Amores et al. 2014; Chojnowski et al. 2016; Davis and Stellwag 2010; Guidato et al. 2003; Hogan et al. 2004; Kameda 2009; Le Pabic et al. 2009; Lee et al. 2013; Manzanares et al. 1999, 2001; McNulty et al. 2005; Mungpakdee et al. 2008b; Oulion et al. 2011; Watari-Goshima and Chisaka 2011; Woltering et al. 2009).

Comparative genomic DNA sequence analysis

The *Hoxa4-Hoxa3* intergenic region has been functionally tested in mice and chickens (Manzanares et al. 1999, 2001). We observed that this region was conserved with all non-teleost fish *Hoxa4-Hoxa3* intergenic sequences analyzed (Fig. 2 and 3). This includes tetrapod species with characterized *Hoxa3* gene expression patterns, including mice, chickens, brown anole lizards, corn snakes, and frogs as well as smaller spotted catsharks. This sequence conservation also includes humans (*Homo sapiens*), coelacanths, and spotted gar (Pough et al. 2019). However, no conserved r5/r6 peaks were observed for *hoxa3a* of zebrafish, Japanese medaka, Japanese pufferfish, Nile tilapia, or three-spined sticklebacks, or for *hoxa3a*α and *hoxa3a*β of Atlantic salmon when compared to the mouse reference sequence (Fig. 2). Further, our Clustal sequence analysis showed that the specific CREs characterized in the mouse and chicken r5/r6 enhancer region, namely Kreisler, Prep/Meis, and both Hox/Pbx sites, are highly conserved among tetrapods, coelacanths, spotted gar, and smaller spotted catsharks (Fig. 3). These results suggest that divergent genomic

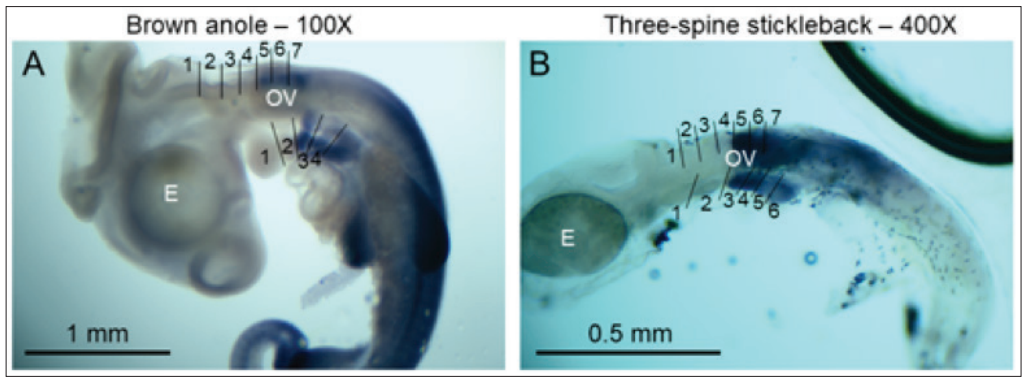

Figure 1. Whole mount *in situ* hybridization results of brown anole *Hoxa3* (A) and three-spine stickleback *hoxa3a* (B). Embryos shown in this figure are representative of replicate embryos. Embryos of both species were mounted such that the anterior side is facing left, and the lateral side is facing the reader. Brown anole lizard and three-spine stickleback embryos were photographed at 100X and 400X magnification, respectively. Numbers on dorsal and ventral sides of the embryonic heads correspond to rhombomeres and pharyngeal arches, respectively. Lines correspond to boundaries between rhombomeres and pharyngeal arches. Scale bars represent 1 mm for brown anole and 0.5 mm and three-spine stickleback. E: eye; OV: otic vesicle.

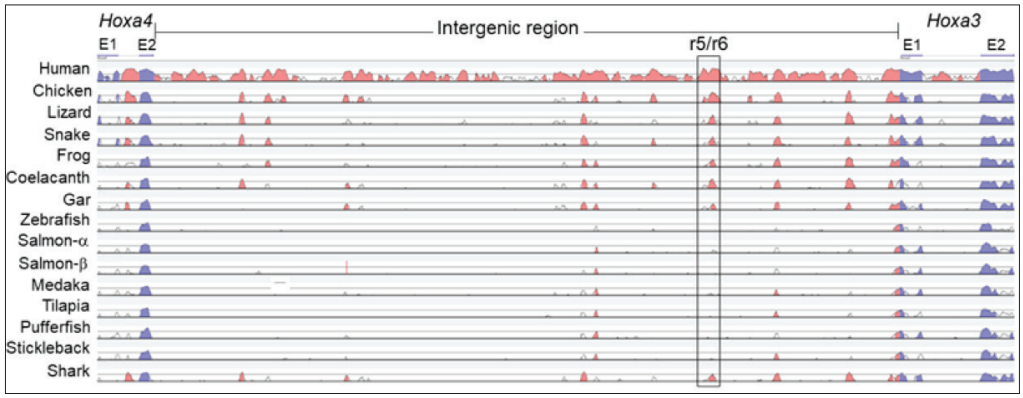

Figure 2. Genomic DNA sequence analysis using mVISTA of the *Hoxa4-Hoxa3* intergenic region. All peaks correspond to DNA sequences that are conserved with the mouse genomic DNA reference sequence. Blue-shaded peaks correspond to exons of *Hoxa4* and *Hoxa3* of tetrapods, coelacanths, and smaller spotted catsharks and *hoxa4a* and *hoxa3a*, *hoxa4a*α and *hoxa3a*α, and *hoxa4a*β and *hoxa3a*β of teleost fish that are at or above 70% sequence conservation identity with respect to the mouse genomic reference sequence. Red-shaded peaks correspond to noncoding DNA sequences that are also at or above 70% sequence conservation identity. Uncolored peaks correspond to coding or noncoding sequences that are below 70% sequence conservation identity. The region containing the r5/r6 enhancer was identified using mVISTA and is labeled in the figure. This region was not conserved for the teleost fish sequences, as the peaks for this region were lacking or minimal in size. Other conserved noncoding DNA peaks were not analyzed as these regions did not make up the r5/r6 enhancer. E1 and E2 correspond to exons 1 and 2 of *Hoxa4* and *Hoxa3*, *hoxa4a* and *hoxa3a*, *hoxa4a*α and *hoxa3a*α, and *hoxa4a*β and *hoxa3a*β.

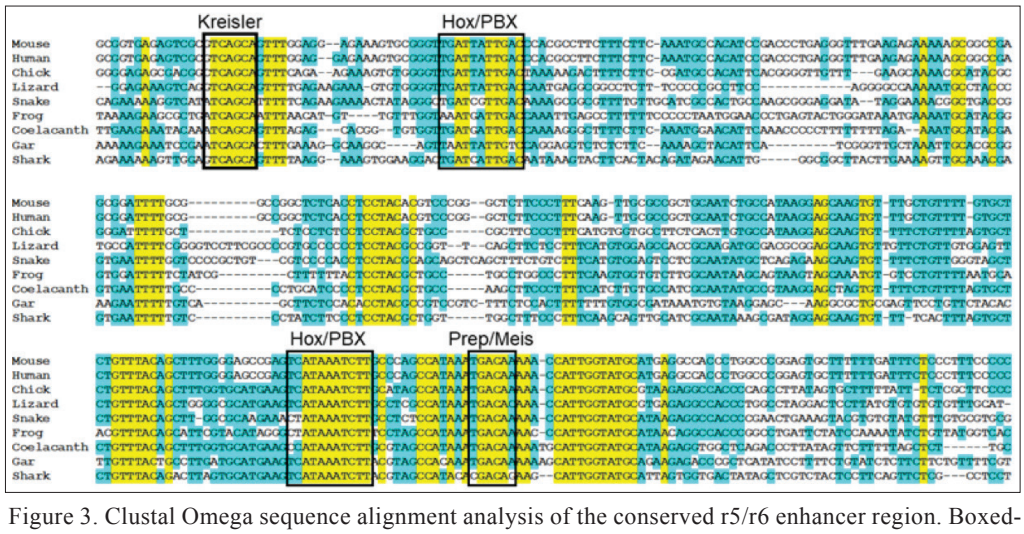

Figure 3. Clustal Omega sequence alignment analysis of the conserved r5/r6 enhancer region. Boxed-in sequences correspond to CREs that were functionally characterized in mice and chickens. Yellow coloring corresponds to 100% sequence conservation at specific nucleotide sites across all species analyzed. Blue coloring corresponds to nucleotides at specific sites that show less than 100% sequence conservation but greater than 50% sequence conservation among all species analyzed. Uncolored nucleotides correspond to less than 50% sequence conservation. Teleost fish sequences were not incorporated into this analysis as there was little to no conservation with the r5/r6 enhancer region between teleost fishes and other gnathostome vertebrates.

sequences are necessary for directing the expression of *hoxa3a*, *hoxa3aα,* and *hoxa3aβ* in the posterior rhombomeres and PAs of teleost fishes.

Discussion

Our expression pattern analyses of brown anole *Hoxa3* and three-spine stickleback *hoxa3a* show that these genes are conserved in their expression with orthologous genes of evolutionarily divergent gnathostomes, including mice, chickens, corn snakes, frogs, smaller spotted catsharks, zebrafish, Japanese medaka, Nile tilapia, Japanese pufferfish, and Atlantic salmon (Amores et al. 2014; Chojnowski et al. 2016; Davis and Stellwag 2010; Guidato et al. 2003; Hogan et al. 2004; Kameda 2009; Le Pabic et al. 2009; Lee et al. 2013; Manzanares et al. 1999, 2001; McNulty et al. 2005; Mungpakdee et al. 2008b; Oulion et al. 2011; Watari-Goshima and Chisaka 2011; Woltering et al. 2009). The conserved expression patterns within the embryonic head suggests that these genes are involved in conserved functional roles in hindbrain and pharyngeal arch development (Chojnowski et al. 2016, Gordon 2018).

In contrast to the conservation of expression among these genes, our comparative genomic assays show that teleost fishes have diverged with respect to gene regulation of *hoxa3a*, *hoxa3aα* and *hoxa3aβ*. Specifically, while tetrapods, coelacanths, sharks, and spotted gar show conservation in sequence with the r5/r6 enhancer region upstream of *Hoxa3*, this region was observed to be degraded in conservation or completely lacking upstream of *hoxa3a*, *hoxa3aα* and *hoxa3aβ* of teleost fishes. These results along with the conserved gene expression patterns of teleost *hoxa3a* genes suggest that the location and/or sequence organization of these elements are divergent for teleost fishes.

In line with our results, previous studies have shown that the intergenic regions of *Hox* clusters containing putative CREs exhibit very little variation between chondrichthyan and sarcopterygian lineages, thereby suggesting high evolutionary constraint of ancestral CREs for these lineages (Raincrow et al. 2011). By contrast, relaxed selective constraint for orthologous regions in the actinopterygian lineages, including teleost fish, was detected, such that many ancestral *Hox* intergenic CRE regions found in chondrychthyans and sarcopterygians were lost or displaced (Raincrow et al. 2011). Further, specific to the lineage leading to teleosts, the whole-genome duplication event would have given rise to two *Hoxa3* genes, *hoxa3a* and *hoxa3b*. The presence of two redundant *Hoxa3* genes in this lineage may have allowed for relaxed constraint within intergenic genomic sequences upstream of these duplicates.

The subsequent loss of *hoxa3b* in the lineage leading to teleost fishes may have reinforced the selective constraint on the remaining *hoxa3a*, such that while the genomic makeup of the CREs directing *hoxa3a* gene expression have diverged, the ancestral *Hoxa3* expression pattern within the rhombomeres and PAs has been retained. Similar results were observed for *hoxb2a* of teleost fish, wherein the location and orientation of several rhombomere expression directing CREs were divergent from those of *Hoxb2* of tetrapods, while the ancestral *Hoxb2* expression pattern has been retained for *hoxb2a* (Scemama et al. 2002). Alternatively, the relaxed constraint on *hoxa3a* following the teleost fish-specific genome duplication may have also allowed for the complete loss of the ancestral r5/r6 CREs and the repurposing of other enhancer regions to maintain conserved anterior limits of *hoxa3a*, *hoxa3aα* and *hoxa3aβ* expression in r5 and PA3. Interestingly, results for this scenario were observed for the regulation of *Otx2* gene expression by the anterior neuroectoderm (AN) and forebrain/midbrain (FM) enhancers, which regulate *Otx2* expression within

the anterior neuroectoderm at earlier and later stages during embryonic development, respectively (Kurokawa et al. 2006). Where *Otx2* of tetrapods and chondrichthyan fish utilizes both enhancer regions, the AN enhancer has been lost for *otx2a* and *otx2b* in teleost fishes (Kurokawa et al. 2006). Further, the FM enhancer region exhibits both AN and FM activity for teleosts, and the AN activity is driven by CREs that are derived in telesost fish (Kurokawa et al. 2006).

Aside of the r5/r6 enhancer, several enhancer regions have been defined in the mouse and chicken model systems that drive *Hoxa3* expression within other embryonic tissues, including somites, lateral plate mesoderm, and tail bud (Manzanares et al. 1999). Future functional assays must be employed to determine if any of these regions or other surrounding genomic sequences have been repurposed to drive teleost *hoxa3a*, *hoxa3a*α or *hoxa3a*β to be expressed within the posterior rhombomeres and PAs during embryonic development.

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