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Gene Expression Pattern Analysis of Anterior Hox Genes during Zebrafish (Danio rerio) Embryonic Development Reveals Divergent Expression Patterns from Other Teleosts

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GENE EXPRESSION PATTERN ANALYSIS OF ANTERIOR *HOX* GENES DURING ZEBRAFISH (*DANIO RERIO*) EMBRYONIC DEVELOPMENT REVEALS DIVERGENT EXPRESSION PATTERNS FROM OTHER TELEOSTS

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ABSTRACT

The regional identity of organs and organ systems along the anterior-posterior axis during embryonic development is patterned, in part, by *Hox* genes, which encode transcription factor proteins that activate or repress the expression of downstream target genes. Divergent nested *Hox* gene expression patterns may have had a role in facilitating morphological divergence of structures, such as the pharyngeal jaw apparatus, among evolutionarily divergent teleost fishes. Recent studies from several evolutionarily divergent teleosts, such as the Japanese Medaka (*Oryzias latipes*) and the Nile Tilapia (*Oreochromis niloticus*), have shown the presence of divergent expression patterns of several *Hox* genes within paralog groups 2–5 between these species. Specifically, these expression patterns were documented in the pharyngeal arches, which give rise to the pharyngeal jaw apparatus. While the expression patterns of several Zebrafish (*Danio rerio*) *Hox* genes that are orthologous to those of Medaka and Tilapia have been documented within the developing hindbrain and pharyngeal arches, many still have yet to be documented, especially within the pharyngeal arches during the postmigratory cranial neural crest cell stages. Here, we present the expression patterns of six Zebrafish *Hox* genes, *hoxc3a*, *d3a*, *a4a*, *d4a*, *b5a,* and *c5a*, within the pharyngeal arches during a postmigratory cranial neural crest cell stage and compare them to their orthologous genes of Medaka and Tilapia at similar stages. We show that while *hoxc3a*, *d3a*, and *c5a* of Zebrafish are absent from the pharyngeal arches, *hoxa4a*, *d4a*, and *b5a* show divergent expression patterns from their orthologs in Medaka and Tilapia. These observed divergences may be, in part, responsible for the divergent pharyngeal jaw apparatus structures exhibited by these fishes.

Keywords: *Danio rerio*, *Hox* gene expression, pharyngeal arches, embryonic development

INTRODUCTION

Anterior-posterior patterning of organs and organ systems during animal embryonic development is largely determined by the nested expression patterns of *Hox* genes, which are evolutionarily conserved (McGinnis and Krumlauf 1992). *Hox* genes encode for transcription factors and are organized in clusters of up to 14 genes within the genome of chordates and are expressed along the anterior-posterior axis during embryonic development collinear with their position within clusters (Ferrier et al. 2000; Holland and Garcia-Fernandez 1996; McGinnis and Krumlauf 1992; Powers and Amemiya 2004). Clustered *Hox* genes have provided some of the first lines of evidence for multiple genome duplications, with one cluster being present in chordates, four in tetrapods, seven to eight in most teleosts, and even thirteen in salmoniform fishes (Amores et al. 1998, 2004; Hoegg et al. 2007; Moghadam et al. 2005; Mungpakdee et al. 2008a; Prince 2002; Stellwage 1999). Independent gene loss after genome duplication has led to *Hox* paralog groups that differ in the number of genes among evolutionarily divergent species, especially teleosts (Amores et al. 1998, 2004; Davis et al. 2008; Davis and Stellwag 2010; Kurosawa et al. 2006; Le Pabic et al. 2007, 2009, 2010; Lyon et al. 2013; Mungpakdee et al. 2008a,b; Scemama et al. 2006; Soshnikova et al. 2013; Tümpel et al. 2006). Further, independent mutations to *cis*-regulatory elements that regulate *Hox* gene expression after genome duplication and species diversification have generated variable expression patterns and, subsequently, variable functions between paralogous genes and orthologous genes within and among evolutionarily divergent species (Amores et al. 2004; Davis et al. 2008; Davis and Stellwag 2010; Davis et al. 2016; Hunter and Prince 2002; Le Pabic et al. 2007, 2009, 2010; Lyon et al. 2013; Mungpakdee et al. 2008b; Scemama et al. 2006). These factors may, in part, have been a major driving force in the evolution of diverse morphological features observed among evolutionarily divergent species (Caroll 2008; Davidson et al. 2006; Soshnikova et al. 2013).

In teleost fishes, the pharyngeal jaw apparatus develops from the cranial neural crest cells of the posterior pharyngeal arches (pharyngeal arch 3–7) and constitutes a set of internal jaws that is distinct from the oral jaws (Kimmel et al. 2001; Nelson et al. 2016; Schaeffer and Rosen 1961; Liem 1973). The pharyngeal jaw apparatus shows high morphological variability among evolutionarily divergent teleost fishes, and such variability may have allowed for divergent mechanisms of nutrient extraction from diverse niches as well as an explosive adaptive radiation of teleost fishes into the most species-rich of all vertebrate groups (Nelson et al. 2016; Schaeffer and Rosen 1961; Liem, 1973). The difference in structure of the bony elements of the pharyngeal jaw apparatus among these teleosts may be due to divergent nested *Hox* gene expression patterns within the posterior pharyngeal arch, which have been shown experimentally to function in patterning the cranial neural crest cells into specific cartilaginous structures within the pharyngeal arch (Crump et al. 2006; Minoux et al. 2009; Santagati et al. 2005). Unfortunately, very few studies, outside of those using the Japanese Medaka (*Oryzias latipes*, order Beloniformes; Davis et al. 2008; Davis and Stellwag 2010) and Nile Tilapia (*Oreochromis niloticus*, order Perciformes; Le Pabic et al. 2007, 2009; Lyon et al. 2013), describe the nested *Hox* expression patterns within the posterior pharyngeal arch. While the expression patterns of *hoxa2b*, *b2a*, *a3a*, *b3a*, *b4a*, *a5a*, and *b5b* within the pharyngeal arches have been documented for Zebrafish (*Danio rerio*, order Cypriniformes; Hunter and Prince, 2002; Hogan et al. 2004; Miller et al. 2004; Hortopan et al. 2011; Thorsten et al. 2004), an analysis of several other *Hox* genes is required to

gain a better understanding on how divergent bony structures of the pharyngeal jaw apparatus develop within evolutionarily divergent teleost fishes.

In this study, we present the expression patterns of Zebrafish *Hox* genes *hoxa4a*, *d4a*, and *b5a* within the pharyngeal arches and compare them to their strict orthologs of Medaka and Tilapia. Expression patterns are reported at 48 hours post fertilization (hpf), which is a postmigratory cranial neural crest cell development stage that occurs just prior to chondrogenesis (Schilling et al. 1994). Several studies have shown that *Hox* gene expression is required in the pharyngeal arches up until chondrogenesis of postmigratory cranial neural crest cells for proper craniofacial skeleton development (Baltzinger et al. 2005; Grammatopoulos et al. 2000; Gendron-Maguire et al. 1993; Pasqualetti et al. 2000; Rijli et al. 1993; Santagati et al. 2005). In particular, Santagati et al. (2005) showed that cranial neural crest cells exhibit plasticity late into their development of the craniofacial bones and thus require *Hox* gene expression to be maintained in the pharyngeal arches prior to cartilage formation. Further, it has been shown that *Hox* gene expression becomes downregulated beyond chondrogenesis of the craniofacial skeleton, and ectopic expression of such genes within the pharyngeal arches at this stage impairs cartilage development (Massip et al. 2007). We also determined if *hoxc3a*, *d3a*, and *c5a* were expressed in the pharyngeal arches at this developmental stage. While we did not observe any expression of *hoxc3a*, *d3a*, or *c5a* within the pharyngeal arches, we found that Zebrafish *hoxa4a*, *d4a*, and *b5a* showed divergent pharyngeal arch-specific expression patterns from their strict orthologs of Medaka, Tilapia, or both. Finally, we offer a full comparison of *Hox* paralog group 2–5 gene expression within the rhombomeres of the hindbrain and pharyngeal arches at the postmigratory cranial neural crest cell stage between Zebrafish, Medaka, and Tilapia. This comparison is based off of our results and results recovered from the literature.

METHODS AND MATERIALS

Forty-eight hpf embyros that were paraformaldehyde-fixed, dechorionated, and stored in methanol were kindly donated by Dr. Pierre Le Pabic of University of North Carolina, Wilmington (IACUC protocol: #A3416–01). At this stage, the pharyngeal arches are well segmented and easily distinguishable. Plasmid DNAs containing the *hoxc3a*, *d3a*, *a4a*, *d4a*, *b5a*, and *c5a* sequences were obtained from Addgene (Cambridge, Massachusetts) and were originally developed and deposited by Prince et al. (1998a,b,c). 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 Leica DM750 compound microscope with an attached Leica ICC50 digital camera system. Images were processed using Adobe Photoshop.

RESULTS

Zebrafish *Hoxc3a*, *D3a*, and *C5a* Are Not Expressed in the Pharyngeal Arches at 48 Hours Postfertilization

We did not observe any pharyngeal arch-specific expression of Zebrafish *hoxc3a*, *d3a*, or *c5a* within the pharyngeal arches at 48 hpf (data not shown). A similar lack of expression in the pharyngeal arches was observed for all three orthologs in Medaka and for *hoxc3a*

and *c5a* in Tilapia (Davis and Stellwag 2010; Le Pabic et al. 2009; Lyon et al. 2013). By contrast, Tilapia showed *hoxd3a* expression in pharyngeal arches 4 and 5 (Le Pabic et al. 2009; Lyon et al. 2013).

Zebrafish *Hoxa4a* Gene Expression Pattern

Zebrafish *hoxa4a* was observed to be expressed in pharyngeal arches 6 and 7 at 48 hpf (Figure 1A). This expression pattern was shown to be divergent from its strict ortholog of both Medaka and Tilapia, both of which were shown to be expressed in pharyngeal arches 5–7 (Davis and Stellwag 2010; Le Pabic et al. 2009; Lyon et al. 2013).

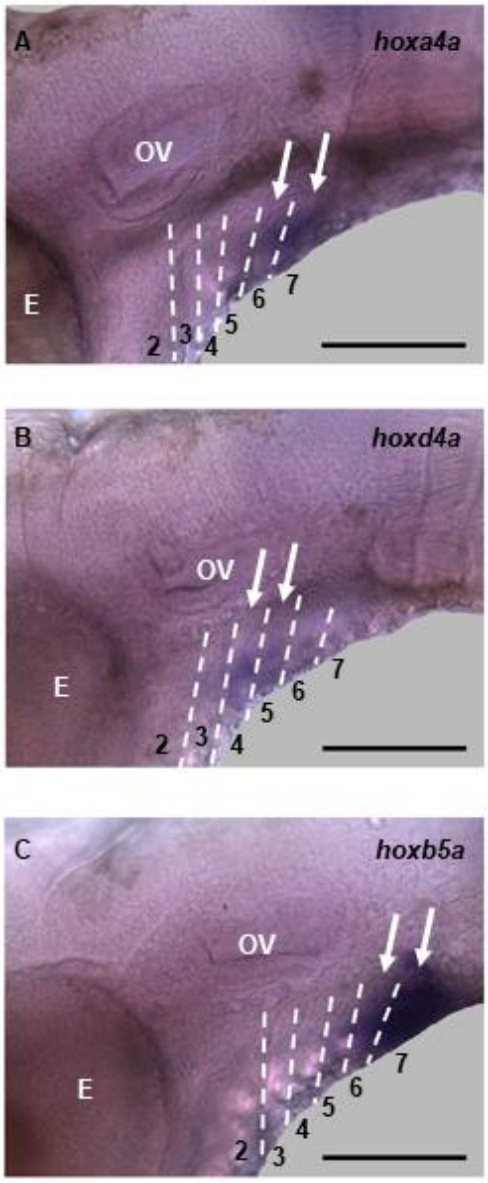

Figure 1. Whole mount in situ hybridization analysis of Zebrafish *hoxa4a* (A), *hoxd4a* (B), and *hoxb5a* (C) at 48 hpf. All embryos were mounted with their anterior sides facing left and lateral sides facing the reader. Numbers on or below the ventral side of the embryo correspond to the pharyngeal arches. Arrows correspond to *Hox* gene-expressing arches. E, eye; OV, otic vesicle. Scale bars equal 0.1 mm.

Zebrafish *Hoxd4a* Gene Expression Pattern

Zebrafish *hoxd4a* was observed to be expressed in pharyngeal arches 4 and 5 at 48 hpf (Figure 1B). This expression pattern was shown to be divergent from that of its strict orthologs in both Medaka and Tilapia, both of which were shown to be expressed in pharyngeal arches 4–7 (Davis and Stellwag 2010; Le Pabic et al. 2009; Lyon et al. 2013).

Zebrafish *Hoxb5a* Expression Pattern

Zebrafish *hoxb5a* was observed to be expressed in pharyngeal arches 6 and 7 at 48 hpf (Figure 1C). This expression pattern was shown to be similar to that of Tilapia but divergent from Medaka's, which was observed to be expressed in pharyngeal arches 5–7 (Davis and Stellwag 2010; Le Pabic et al. 2009; Lyon et al. 2013).

DISCUSSION

Our expression pattern analysis of Zebrafish *hoxc3a*, *d3a*, *a4a*, *d4a*, *b5a*, and *c5a*, in conjunction with data from several previous gene expression analyses in Zebrafish (Hunter and Prince 2002; Hogan et al. 2004; Miller et al. 2004; Hortopan et al. 2011; Thorsten et al. 2004), Medaka (Davis et al. 2008; Davis and Stellwag, 2010), and Tilapia (Le Pabic et al. 2007, 2009; Lyon et al. 2013) provides the first comparative study of nested *Hox* paralog group 2–5 gene expression patterns in the pharyngeal arches between three evolutionarily divergent teleost fishes that exhibit divergent pharyngeal jaw apparatus structures. The postmigratory cranial neural crest cells of the pharyngeal arches of Zebrafish express *hoxa2b* and *b2a* in pharyngeal arch 2, *hoxa2b*, *a3a*, and *b3a* in pharyngeal arch 3, *hoxa2b*, *a3a*,, *b3a*, *b4a*, and *d4a* in pharyngeal arch 4, *hoxa2b*, *a3a*, *b3a*, *b4a*, and *d4a* in pharyngeal arch 5, and *hoxa2b*, *a3a*, *b3a*, *a4a*, *b4a*, *a5a*, *b5a*, and *b5b* in pharyngeal arches 6 and 7 (Figure 2). The loss of several *Hox* genes to pseudogenes has been an important factor in generating divergence in nested *Hox* gene expression in both the hindbrain and pharyngeal arches among all three species. Specifically, while Medaka has lost *hoxa2b* to pseudogenization (Davis et al. 2008), Zebrafish has lost *hoxa2a*, *b3b*, and *d4b* (Amores et al. 1998). Genes with different expression patterns from those in Zebrafish include *hoxb2a* of both Medaka and Tilapia, which are expressed in pharyngeal arches 2–7 (Davis et al. 2008; Le Pabic et al. 2007), *hoxd3a* of Tilapia, which is expressed in pharyngeal arches 4 and 5 (Le Pabic et al. 2009; Lyon et al. 2013), *hoxa4a* and *b4a* of both Medaka and Tilapia, which are both expressed in pharyngeal arches 5–7 (Davis and Stellwag 2010; Le Pabic et al. 2009; Lyon et al. 2013), *hoxd4a* of both Medaka and Tilapia, which are both expressed in pharyngeal arches 4–7 (Davis and Stellwag, 2010; Le Pabic et al. 2009; Lyon et al. 2013), *b5a* of Medaka, which is expressed in pharyngeal arches 5–7 (Davis and Stellwag 2010), and *hoxb5b* of both Medaka and Tilapia, which are both expressed in pharyngeal arch 7 (Davis and Stellwag 2010; Le Pabic et al. 2009; Lyon et al. 2013). Given the divergence in *Hox* gene content and pharyngeal arch-specific expression patterns, it is tantalizing to suggest that these differences may have, in part, provided the molecular mechanisms necessary to pattern the morphologically divergent pharyngeal jaw apparatus structures among these evolutionarily divergent teleosts. In support, several studies have suggested the presence of a "*Hox* code", or a nested and segment-specific combination of expressed *Hox* gene products along the anterior-posterior axis, that patterns the identities of the pharyngeal

arches, and thus their morphological derivatives (Minoux et al. 2007; Parker et al. 2018). Further, the combinatorial code of *Hox* gene products has been shown to be patterned, in part, by specific auto- and cross-regulatory roles among *Hox* genes in specific pharyngeal arches (see Parker et al. 2018). Divergent pharyngeal arch-specific *Hox* codes among evolutionarily divergent teleost fishes may therefore be due to variation in *Hox* gene regulatory networks. Developmental genetic studies in Zebrafish and Nile Tilapia have shown the presence of divergent mechanisms in directing the morphogenesis of the

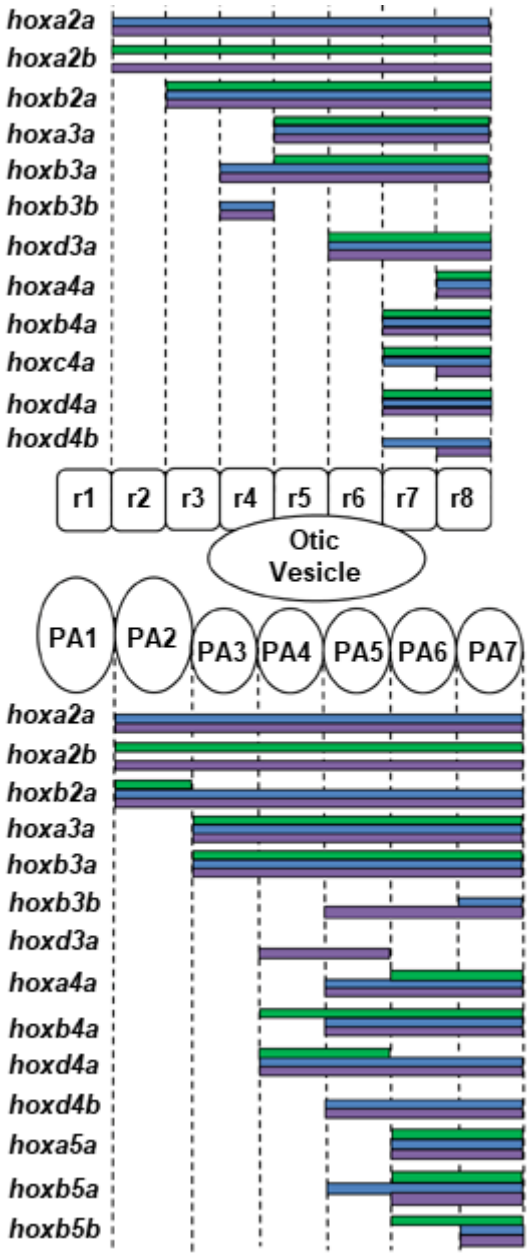

Figure 2. The comparative combinatorial code of *Hox* paralog group 2–5 gene expression in the hindbrain and pharyngeal arches during postmigratory cranial neural crest cell stages among Zebrafish (green bars), Medaka (blue bars), and Tilapia (purple bars). All rhombomere and most pharyngeal arch expression patterns are referenced from the literature. *Hoxc4a* is not shown for the pharyngeal arches for all three

teleosts, because the expression pattern is not known in Zebrafish during postmigratory cranial neural crest cell stages. PA, pharyngeal arch; r, rhombomere.

skeletal derivatives from the second pharyngeal arch (Hunter and Prince 2002; Le Pabic et al. 2010). In Zebrafish, only two *Hox* genes are expressed in this segment, *hoxa2b* and *b2a*, and these genes were shown to function redundantly in patterning the segment's identity, such that the knockdown of both genes is required to produce a homeotic transformation of the pharyngeal arch 2-derived bones into those that resemble the products from pharyngeal arch 1 (Hunter and Prince 2002). These results suggest that both genes are involved in cross- and auto-regulatory mechanisms within this arch. By contrast, Nile Tilapia shows three genes expressed in the second pharyngeal arch, *hoxa2a*, *a2b*, and *b2a*, and only *hoxa2a* is required to be knocked down for a full homeotic transformation (Le Pabic et al. 2010). The knockdown of *hoxa2b* or *b2a* resulted in only slightly altered bony structures (Le Pabic et al. 2010). Further, the knockdown of Nile Tilapia *hoxa2a* results in reduced expression of itself in the pharyngeal arches and *hoxb2a* in the second pharyngeal arch, while the loss of *hoxb2a* results in reduced expression levels of *hoxa2a* and *a2b* from just the second pharyngeal arch (Le Pabic et al. 2010). Disparate combinations of *Hox* gene expression patterns between evolutionarily divergent teleosts may therefore have led to divergent genetic regulatory networks within homologous pharyngeal arches among species and overall divergence in bony elements derived from these embryonic modules. In cichlids and several other perciform fishes, the pharyngeal jaw apparatus skeleton includes fused lower jaw bones and a diarthrotic articulation between elements of the upper jaw with the ventral side of the neurocranium (Liem, 1973; Stiassny and Jensen 1987). In Medaka and other beloniform fishes, specializations of the pharyngeal jaw apparatus include a reduction in the size of the second and third epibranchials (which are derived from the dorsal regions of pharyngeal arches 4 and 5, respectively), an expansion of the articular surface of the fourth epibranchial (a dorsal pharyngeal arch 6 derivative), and the presence of large ventral flanges on the fifth ceratobranchial (a ventral pharyngeal arch 7 derivative; Langille and Hall 1987; Parenti 1987; Rosen and Parenti 1981). Pharyngeal jaw apparatus specializations in Zebrafish and other cypriniform fishes include an enlarged fifth ceratobranchial (a ventral pharyngeal arch 7 derivative) with teeth ankylosed to the bone and the absence of the first pharyngobranchial (a dorsal pharyngeal arch 3 derivative; Nelson et al. 2016). Further investigation of pharyngeal arch-specific *Hox* gene expression patterns of *Hox* paralog group 2–5 genes of other teleosts, such as Stickleback (*Gasterosteus aculeatus*), is necessary to better understand how *Hox* genes shape divergent structures derived from the pharyngeal arches in teleosts.

In addition to the pharyngeal arches, several orthologous *Hox* genes among Zebrafish, Medaka, and Tilapia have been shown to exhibit divergent expression patterns in the rhombomeres of the hindbrain (Davis et al. 2008; Davis and Stellwag 2010; Hunter and Prince 2002; Le Pabic et al. 2007, 2009; Lyon et al. 2013; Moens and Prince 2002; Prince and Lumsden 1994; Prince et al. 1998a,b,c). These genes include *hoxb3a*, which has an anterior limit of expression at the rhombomere 4/5 boundary for Zebrafish and the rhombomere 3/4 boundary for Medaka and Tilapia; and *hoxc4a*, which has an anterior limit at the rhombomere 7/8 boundary for Zebrafish and the rhombomere 6/7 boundary for Tilapia (Figure 2). The divergent rhombomere and pharyngeal arch-specific expression patterns of several orthologous *Hox* genes among Zebrafish, Medaka, and Tilapia may be the result of divergence of orthologous genomic sequences corresponding

to the *cis*-regulatory elements that direct their expression. Numerous studies have shown that rhombomere and pharyngeal arch-specific *Hox* gene expression requires complex interactions between multiple *cis*-regulatory elements (Amin et al. 2015; Davis et al. 2016; Maconochie et al. 1999, 2001; McEllin et al. 2016; Parker et al. 2018; Tümpel et al. 2006, 2009). Mutations to such *cis*-regulatory elements may have altered the interactivity of their respective transcription factors, and thus altered the spatiotemporal *Hox* expression patterns within these embryonic compartments. Recently, Davis et al. (2016) showed that slight differences in relatively short sequences of genomic DNA can yield highly divergent expression patterns between paralogous sequences. Specifically, while a 89-bp intergenic region upstream of Medaka *hoxa2a* was shown to direct reporter gene expression in rhombomere 4 and pharyngeal arches 2–7, the paralogous 88-bp sequence upstream of the pseudogene Medaka *hoxa2b* drives reporter gene expression in rhombomeres 3–8 and pharyngeal arches 2–7 (Davis et al. 2016). Further, comparative genomic sequence analysis shows that the divergence of reporter gene expression is driven by the presence of only 33 bp differences between the two paralogous sequences (Davis et al. 2016). Similar studies involving paralogous sequences of *hoxa2a* and *a2b* of Japanese puffer also show that mutations at *cis*-regulatory elements can lead to highly divergent gene expression patterns (McEllin et al. 2016; Tümpel et al. 2006). In order to determine the molecular mechanisms that generate divergent *Hox* expression patterns in teleost rhombomeres and pharyngeal arches and, ultimately, the divergent morphological features that arise from these embryonic domains, reporter gene expression studies should be performed using orthologous sources of genomic DNA from multiple evolutionarily divergent teleosts.

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