## HOX CLUSTER INTERGENIC SEQUENCE EVOLUTION

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# ABSTRACT OF THE DISSERTATION <br> HOX GENE CLUSTER INTERGENIC SEQUENCE EVOLUTION by JEREMY DON RAINCROW 

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The Hox gene cluster system is highly conserved among jawed-vertebrates. Specifically, the coding region of Hox genes along with their spacing and occurrence is highly conserved throughout gnathostomes. The intergenic regions of these clusters however are more variable. During the construction of a comprehensive non-coding sequence database we discovered that the intergenic sequences appear to also be highly conserved among cartilaginous and lobe-finned fishes, but much more diverged and dynamic in the ray-finned fishes. Starting at the base of the Actinopterygii a turnover of otherwise highly conserved non-coding sequences begins. This turnover is extended well into the derived ray-finned fish clade, Teleostei. Evidence from our population genetic study suggests this turnover, which appears to be due mainly to loosened constraints at the macro-evolutionary level, is highlighted by evidence of strong positive selection acting at the micro-evolutionary level. During the construction of the non-coding sequence database we also discovered that along with evidence of both relaxed constraints and positive selection emerges a pattern of transposable elements found within the Hox gene cluster system. The highly conserved Chondrichthyes and Sacropterygii Hox gene clusters have an invasion of type I transposons whereas the Actinopterygii Hox gene clusters have an invasion of type II transposons. Specifically,
the Tc 1 transposon is found throughout the ray-finned fishes Hox gene clusters and is highlighted by the presence of two intact Tc1 transposons in and adjacent to bichir's Hox gene clusters. Expression in human cell lines suggests that at least one of these Tc1 transposons are active. This combined with simulations ran in our lab point to transposons having a role in past and on-going restructuring of ray-finned fishes genomes.

These findings help shed light on the possible genomic changes that occurred and are occurring within the ray-finned fish clade that help shed light on their past and present species radiations.

## Dedication

To
Jim and Patsy Brown
for their tireless support throughout my education
To

## Wilder and Angelica Pajares

for giving me a home away from home
To
Maricruz Pajares
for keeping my spirits high
To

## Kim Harris

for starting me on the right path

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## Introduction- The Unrivaled Teleosts and the Model Meta-Gene

### 1.1 The Unrivaled Teleosts

### 1.1.1 Speciose Teleosts

Teleost fishes (division: Teleostei) are a highly successful clade both from the species and morphological perspective. Teleosts currently contain $\sim 27,000$ species that comprise nearly one-half of all jawed-vertebrates (superclass: Gnathostomata) and 99\% of all ray-finned fishes (class: Actinoptergyii; Nelson 2006). These species are divided into 38 orders with 426 families and 4,084 genera (Nelson 2006).

Along with this taxonomic success is a stunning array of adaptations. Some of the more striking adaptations include fishes that have secondarily lost paired appendages (true eels; Anguilliformes), have the ability to inflate their bodies to twice the normal size (pufferfishes; Tetraodontidae), have the ability to create their own light (flashlight fish, Photoblepharon palpebratus) and even produce electrical fields strong enough to stun or kill (electric ray; Torpedinidae, electric eel; Electrophoridae, stargazer; Uranoscopidae). Teleosts range in size from a 7.9 mm cyprinid (Paedocypris progenetica, Kottelat et al. 2006) to a 2-ton ocean sunfish (Mola mola, McCann 1961).

The anterior dorsal fin is an example of the variety of adaptations that can be seen even in a single anatomical structure. This single fin has been modified into a lure (Lophiidae), camouflage (Antennariidae), suction disks (Echeneidae), and venom delivery (Synanceja, Trachinidae, Thalassophryninae). As a prime example of the oddity of adaptation seen in teleosts; a recent and very unique phenotype was discovered in the Monterey Bay off the coast of California. A close relative of salmonids, Macropinna microstoma is an opisthoproctid fish with a clear skull and rotating tubular
eyes that let it take in the dim light from above as well as perform its front-facing predatory role (Robison and Reisenbichler 2008).

Along with anatomical diversity, teleosts also maintain increased variation at the cellular level. One example is coloration. Teleost fishes have a highly complex pigmentation patterning system that involves many pigment cell types known as chromatophores which are all derived from the neural crest. They come in 6 varieties including melanophores (black/brown), xanthophores (yellow/orange), iridophores (iridescent), erythrophores (red), leucophores (white) and even cyanophores (blue; Goda and Fujii 1995). This is in stark contrast to the single pigment cell type in mammals and birds (melanocytes; black/brown/yellow). This increase in chromatophores in teleosts is thought to be partly due to the duplication of color gene pathways both from whole genome duplications as well as lineage specific gene duplications (Braasch et al. 2006, 2009).

### 1.1.2 Teleost Specific Morphology

Teleost fishes have undergone rapid speciation coincident with an array of adaptive morphologies (Alfaro et al. 2009). They are one of if not the most diverse and successful extant vertebrate lineages. What conditions in the evolutionary history of actinopterygians (ray-finned fishes) underlie both their speciosity and morphological variability?

One hypothesis is that the acquisition of a few shared, advantageous adaptations early in teleost evolution may be necessary to maintain an ecological foothold while facilitating unique adaptations to a variety of habitats resulting in morphological variability (Figure 3; Hulsey et al. 2006). Such a scenario could lead to the phenomenon
of adaptive morphological plasticity which we define as a species or group of species that appears to rapidly adapt to a variety of habitats. This plasticity is exemplified by the filling of several ecological niches by cichlid fishes in two separate occasions in Lake Malawi, Lake Tanganyika and Lake Victoria of Africa. All of these rapid speciations occurred independently and in different yet short evolutionary time frames (summary, Koblmüller et al. 2008). Do teleost fishes share such a set of advantageous adaptations?

Teleost fishes diverged from there closest living relative $\sim 250$ mya (Figure 2; Hurley et al. 2007). Since that time teleost fishes have evolved into fast, agile fishes able to exploit an almost limitless variety of food sources and habitats. The main adaptations that have contributed to their success have been a reduction in the number and thickness of bones, the amount of bony material in the scales, and fin position and composition allowing for greater flexibility, speed and maneuverability leading to a more dynamic predator/prey relationship (Janvier 1996). Perhaps as important as the increased locomotion is the modification of the feeding structures (Hulsey et al. 2006).

Teleosts have lost many bones in the skull and jaw allowing for the evolution of the extendable front/marginal/oral jaw. The extension of this oral jaw causes negative pressure within the mouth sucking in surrounding water and its contents allowing the fish to better capture prey (Schaeffer and Rosen 1961). However, the oral jaw reduces significantly the bite force and hence the ability of fishes to eat foodstuff containing hard materials such as the chitinous shell of the arthropods and the cellulose cell wall of most plants (Schaeffer and Rosen 1961; Liem 1973). Teleost fishes have developed a second pair of jaws known as pharyngeal jaws that are able to process these harder foodstuffs
(Schaeffer and Rosen 1961). Pharyngeal jaws evolved from a dentin surface on the pharynx and eventually developed musculature (Figure 1).

It is hypothesized that the development of the pharyngeal jaw and subsequent partitioning of duties between the oral and pharyngeal jaws has led to a decoupling of their evolution (Liem 1973). This is known as Liem's hypothesis and is thought to be a major reason for the successful species radiation of the teleost division. It is also possible that novel and useful adaptations such as the pharyngeal jaw may be necessary to allow species/groups, such as the teleosts, to maintain a morphological robustness while allowing unique adaptations to a variety of habitats resulting in adaptive morphological plasticity. This would cause a delay in the rapid speciation events following the key innovation as the new innovation allows the species to move into new habitats and increase in number and then subsequently adapt to those habitats resulting in rapid speciation. Alfaro et al. (2009) found this delay between a pharyngeal jaw innovation and rapid speciation in their labrid (Teleostei:Perciformes) fish.

The pharyngeal jaw and other unique features have allowed teleosts to exploit an almost endless variety of habitats (Liem 1973) including water temperatures of $-2^{\circ} \mathrm{C}$ at the polar caps (DeVries and Wohlschlag 1969) to $41^{\circ} \mathrm{C}$ at the African hot springs (Johnston et al. 1994), from elevations of 3.8 km in the Andean Lake Titicaca (Arratia 1982) to depths of 7.7 km in an ocean trench off the shore of Japan (Jamieson et al. 2009) and various pH , saline, and dissolved oxygen concentrations.

### 1.1.3 Environmental Change

Another driving factor in teleosts success and uniqueness is their environment. Environmental pressures are known to expedite evolution through natural selection. Were there any environmental factors that may have led to the current state of teleosts?

Teleost fishes' fossil record dates back to the beginning of the Triassic period ~250 million years (my) (Hurley et al. 2007). Shortly after, continental drift was occurring that would lead to the break up of the super continent Pangea into Gondwana and Laurasia ( $\sim 150$ mya, Smith et al. 1994). Eventually ( $\sim 100$ mya) Gondwana and Laurasia would break up into the current continents that have maintained a relatively steady shape over the last 65 my . Plate tectonics occurring during the evolution of the teleost fishes were opening up 1,000s of miles of new shoreline for ocean life. This ocean life includes the evolution of modern corals that host nearly one-half of all extant teleost species and is postulated to have been an aide in the overall diversification of the teleost division (Bellwood and Wainwright 2002; Alfaro et al. 2007). Several mass extinctions also occurred during the evolution of the teleost fishes that would lead to less competition for habitat and resources for those surviving individuals. This may have allowed teleosts to reach great numbers that in turn created competition within the clade driving diversity from within while simultaneously suppressing the success/diversification of other clades.

Coincident with the dawning of the teleost fish division was the largest known mass extinction event in the history of Earth, the Permian-Triassic extinction. During this mass extinction event $\sim 95 \%$ of all marine life went extinct. Another major extinction occurred at the end of the Triassic (~200 mya) killing off all marine reptiles except for

Ichthyosaurus. A third mass extinction (Cretaceous-Palaeogene K-P mass extinction) occurred at the end of the Cretaceous period ( $\sim 65$ mya) again resulting in the extinction of all marine reptiles except turtles. Friedman et al. (2010) was able to show that this mass extinction may have led to the Acanthomorph rapid species radiation by allowing the Acanthomorphs to fill the morphological space left void by the extinction of nonteleost vertebrates.

It is unclear if teleosts survived these mass extinctions in greater numbers than other lineages or if they were able to take advantage with the few species that were left. Crow et al. (2005) suggest that the fish specific genome duplication (see section 1.1.4) may have acted as a buffer against extinction for those surviving teleosts. However, Santini et al. (2009) estimated that despite an increase in overall diversification rates in teleosts, teleosts have a 6 times greater extinction rate than non-teleosts gnathostomes. This leads to a one and one-half greater death:birth ratio (of species) in teleosts as opposed to non-teleost gnathostomes. Regardless, teleosts are currently the most successful extant vertebrate lineage. There were also other ray-finned fish lineages that existed prior to the MRCA of living teleosts that were species rich as well that did not survive (Donoghue and Purnell 2005).

Unique and advantageous adaptations, mass extinctions of competitors and vast amounts of new habitat available for niche-filling have helped shape the explosion of teleost fishes' numbers and diversity of species. Are environmental factors and welltimed advantageous adaptations enough or do teleosts fishes have an inherent molecular structure that allows for their success?

### 1.1.4 Fish Specific Genome Duplication

This species and morphological diversity in teleost fishes has been aided by or at least evolved along side not only environmental factors but also changes in genomic architecture and accelerated molecular evolution. A major genomic change that occurred prior to the divergence of extant teleost fishes is a whole genome duplication dubbed the fish specific genome duplication (FSGD; Meyer et al. 1999, 2005). The best evidence that the FSGD is a whole genome duplication comes from synteny maps from the completed genome of the spotted green pufferfish (Tetraodon nigroviridis) showing this taxon has two syntenic regions (paralogons) that correspond to single regions in the human genome (Jaillon et al. 2004). Comparative mapping shows that paralogons of spotted green pufferfish and zebrafish (Danio rerio) are homologous, supporting that the FSGD occurred prior to the divergence of the major teleost clades, Acanthopterygii and Ostariophysi (Woods et al. 2005).

Our labs recent work on Hox genes in the goldeye (Hiodon alosoides) suggests that Osteoglossomorpha, the most basal teleost subdivision, also shares the FSGD with higher teleost fishes (Chambers et al. 2009). Because of the importance of genome duplication in vertebrate evolution (Ohno, 1970; Sidow, 1996) many authors have speculated that the FSGD is directly responsible for the biological diversification (i.e. speciosity) of teleost fishes (Hoegg et al. 2004; Postlethwait et al. 2004; Meyer and Van de Peer 2005).

There are however several lines of evidence that the FSGD is not even coincidental with the teleost species radiation much less a causal agent. Recent
examination of the ray-finned fishes fossil record, shows that there are 11 extinct clades between teleosts and their closest living relatives (Donoghue and Purnell 2005). The authors conclude that the character acquisitions normally attributed as synapomorphies of derived teleost fishes arose gradually in ray-finned fish phylogeny with many acquisitions predating the FSGD. Additionally, many of these extinct clades that have been shown to pre-date the FSGD were species rich themselves. Hence fossil evidence suggests that the FSGD is uncoupled to species richness.

Molecular clock analysis between human (Homo sapiens) and Japanese pufferfish (Takifugu rubripes) estimates the FSGD occurred ~350 mya (Christoffels et al. 2004). Evidence from Hox (Crow et al. 2006), ParaHox (Mulley et al. 2006), and three nuclear, non-Hox genes (Hoegg et al. 2004) supports that the FSGD is coincident with the origin of teleosts in the osteoglossomorphs, after divergence of the bowfin (Amia calva). Bowfin fossils suggest this node is $\sim 250$ million years old (Hurley et al 2007). Importantly, our work with an Osteoglossomorph has shown that possession of a duplicated genome is not sufficient for species richness (Chambers et al. 2009). It is also important to note that the Actinoptyerygian super-orders (Ostariophysi and Acanthopterygii) that contain $93 \%$ of the present-day teleost species diverged $\sim 135$ mya (Benton 2005), much later in ray-finned fish phylogeny than when the FSGD occurred (250-350 mya). Furthermore it has been shown that these species rich super-orders haven't retained many genes as duplicates from the FSGD.

In fact, it is estimated that only $\sim 10 \%$ and $\sim 15 \%$ of paralogous genes in zebrafish (Ostariophysi) and spotted green pufferfish (Acanthopterygii) genomes, respectively are a result of the FSGD (Blomme et al. 2006). Interestingly, for both species the
majority of paralogous genes result from lineage specific duplications ( $\sim 40 \%$ of paralogs in zebrafish and $\sim 17 \%$ in green-spotted pufferfish (Blomme et. al. 2006). Hence these data demonstrate that the FSGD likely did not play a large role in the diversification of teleost fishes but does suggest gene duplication in general may be important.

### 1.1.5 Accelerated Evolution

If not the FSGD what was the major molecular contributor to the diversification of the teleost fishes? It is possible that accelerated evolution due to increased mutation rate and increased genetic recombination are factors.

Both coding and non-coding sequences have been shown to be under accelerated evolution in the teleost division of ray-finned fishes. Robison-Rechavi and Laudet (2001) set out to test the Ohno model that one of two duplicate genes should be less constrained by selection and thus be able to acquire a new function (and presumably an accelerated evolution). What they found was that $16 / 19$ genes in fish regardless of their duplication status showed an overall accelerated evolution when compared to mammals. Growth hormone genes have also been found to be under accelerated evolution (5X more substitutions) in teleosts compared to mammals and this acceleration has been shown to be a product of both relaxation of purifying selection and positive selection (Ryynanen and Primmer 2006).

On a genome-wide scale Jaillon et al. (2004) showed that the higher mutation rate held true for 5,082 protein-coding genes when comparing spotted green pufferfish and Japanese pufferfish divergence to the mammalian proxy of human (Homo sapiens) and the house mouse (Mus musculus). For the $15-25 \%$ of genes retained as pairs there is often an asymmetric acceleration of nucleotide substitution (synonymous and
nonsynonymous) rate in one of the duplicated paralogs (Wagner et al. 2005; Braasch et al. 2006; Brunet et al. 2006; Crow et al. 2006; Steinke et al. 2006). This pattern is also lineage specific and, for Hox genes, also cluster specific (i.e. the HoxAa versus HoxAb clusters of teleosts, Chiu et al. 2004; Wagner et al. 2005).

One signature of molecular adaptation is $\mathrm{Ka} / \mathrm{Ks}>1$ (Nei and Gojobori 1986). This means that there is a greater frequency of non-synonynomous changes than synonynomous changes which implies that non-synonomous changes are preferentially fixed in the population. Investigations of duplicated paralogs in teleost fishes have found one paralog to show evidence of positive Darwinian selection; this asymmetry is species specific (Steinke et al. 2006; Brunet et al. 2006). Interestingly, Brunet et al. (2006) suggest that this asymmetric pattern of Darwinian evolution observed is not a result of changes in selective patterns after the FSGD (Vogel 1998; Meyer and Schartl 1999; Volff 2005). Instead, they propose that genes already under strong selective pressure were preferentially maintained as duplicates. Implying that duplication of genes that are advantageous to adaptation leads to greater adaptive success. This is the first insight into possible molecular mechanisms for the success of the teleosts, the accelerated evolution and possibly positive selection on groups of genes in ray-finned fishes prior to the divergence of teleosts that were also advantageous after their divergence.

This accelerated evolution in the teleost fishes has also been observed in conserved non-coding sequences (CNS). Work done by Chiu et al. (2002) showed that conserved non-coding sequences found between human and horn shark (Heterodontus francisci) HoxA clusters were more often than not absent in the teleost fishes, zebrafish (Danio rerio) HoxAa and HoxAb clusters and striped bass (Morone saxatilis) HoxAa
cluster. Venkatesh et al. (2007) showed that this loss of CNSs occurred at the genome wide scale as well. They were able to show that between human and elephant shark (Callorhinchus milii) there were 4,782 CNSs whereas between human and zebrafish there were only 2,838 and between human and Japanese pufferfish 2,107. This turnover of CNSs in the teleost fish division can be postulated to result in differing expression patterns since CNSs are predicted cis-regulatory elements. However conservation of putative or known cis-regulatory elements does not always coincide with conserved function. Fisher et al. (2006) show human and zebrafish RET-1 cis-regulatory elements have equivalent function in cross-species expression studies, despite no sequence similarity. More research in investigating the effect of cis-regulatory element evolution on gene expression changes in ray-finned fishes is urgently needed.

Although the exact mechanisms of the teleosts' success are unknown their radiation is unmatched among extant vertebrates. It is true that fossil records show other lineages that may have been as successful as teleosts (Donoghue and Purnell 2005), however understanding mechanisms that led to the radiation and quite possibly continued radiation of the extant teleosts will give insight into the environmental, phenotypic, and genetic conditions necessary for successful species radiations.

### 1.2 The Model Meta-Gene

### 1.2.1 Discovery of Hox Genes

In order to gain more insight into possible molecular mechanisms that have contributed to the teleosts rapid species radiation I focus my study on the Hox genes. First, I will give an introduction into Hox gene discovery and evolution as well as their clustered nature.

Homeotic mutations are those that transpose whole body parts or segments. Homeotic mutations were first officially described in 1915 in the fruit fly (Drosophila melanogaster; Bridges and Morgan 1923). In an effort to isolate the genes responsible for these mutations E.B. Lewis devoted a career to studying them in D. melanogaster. In the 1950s, 1960s and 1970s Lewis was able to isolate several clustered homeotic genes that were termed the bithorax complex (BX-C). In a review in 1978 Lewis eloquently laid out a proposal for the rules governing these genes and their expression where each gene had an anterior but not posterior boundary and the expression of each new gene caused the next thoracic or abdominal segment to take on a unique characteristic.

Lewis was incorrect about the number of protein coding genes that were contained within this complex but future insight discovered that micro RNA genes also lie within this complex and help regulate the anterior-posterior patterning in Drosophila and thus implementing them as major contributors to the insect body plan (review, Chopra and Mishra 2006). The BX-C contains 3 homeotic genes (Sanchez-Herrero et al. 1985) or as they are known in Drosophila Hom-C genes (Akam 1989). The rest of the Hom-C genes were described in a cluster by Kaufman et al. (1980) and were coined the Antennapedia complex or ANT-C. Together the BX-C and ANT-C contain 8 Hom-C
genes that are responsible for patterning the anterior-posterior axis of Drosophila melanogaster.

The homeotic genes are now defined by a conserved sequence known as the homeobox. McGinnis et al. (1984a) first described the homeobox as a repetitive DNA sequence that was conserved among genes in the BX-C and ANT-C. This 180bp homeobox codes for the 60 amino acid homeodomain. The homeodomain was considered evidence that homeotic genes were transcription factors due to the homology with the MAT-a1 and MAT- $\alpha 2$ genes of yeast that are known transcription factors that regulate genes responsible for mate type switching (Shepherd et al. 1984). Evidence of homeotic mutations in vertebrates and the discovery of the conserved homeobox sequence led to rapid isolation of homeotic genes in other animals (chicken; Gallus gallus, mouse and human McGinnis et al. 1984b; McGinnis et al. 1984c).

That same year an entire homeobox gene was cloned from the African clawed frog (Xenopus laevis) and it was found to have 55 of 60 amino acids conserved in the homeodomain with D. melanogaster's Antennapedia (Carrasco et al. 1984) and a homeobox gene from mouse was cloned in the same lab (McGinnis et al. 1984c). Because of the diversity of animal types Hox genes were found in, they were looked for in more and more animals to help establish their universality. This was important due to their possible implication as major contributors to the embryological and evolutionary patterning of all animal bauplans.

### 1.2.2 Evolution of Hox Genes

Hox genes are part of a class of helix-turn-helix proteins that are known to bind to DNA and help regulate transcription. The helix-turn-helix motif is one of the most
conserved (oldest) motifs known in living organisms. Giraldo and Diaz-Orejas (2001) found that RepA, which is responsible for DNA replication initiation in Pseudomonas (bacteria) and contains a helix-turn-helix motif, was homologous to Orc4p in Saccharomyces cerevisiae (eukaryote) and Cdc6p in Pyrobaculum aerophilum (archaea) in both sequence and structure making them likely orthologs. This places the origin of the helix-turn-helix motif at or before the most recent common ancestor of all known living organisms.

As mentioned previously, the Hox-related family is determined specifically by the presence of the homeodomain, a 60 amino-acid motif. Proteins containing this domain can be found as deep in animal phylogeny as Cnidaria (Murtha et al. 1991). Although, to be a true Hox family member the gene must be part of a cluster as they are in the BX-C and ANT-C in Drosophila. So far Hox clusters have been discovered in various states of clustering in both protostomes and deuterostomes, which would date the ancestral Hox cluster as far back as the origin of the Bilateria (Figure 2).

The Radiata (Cnidaria and Ctenophora) contain Hox-related genes but there is no decisive proof that they are clustered in the genome (review; Aboobaker and Blaxter 2003). Among Bilateria the oldest known members to contain Hox genes would be the phylum Acoelomorpha. Hox gene orthologs have been found in both classes of Acoelomorpha, Acoela (Cook et al. 2004) and Nemertodermatida (Jimenez-Guri et al. 2006). Hox genes have also been found in the other basal Bilaterian phylum, Chaetognatha (Papillon et al. 2003). Again it is important to point out that there is no indication that these Hox genes are clustered in these sister groups to the superphyla of Protostomia and Deuterstomia.

In protostomes Hox genes have been found in the phyla Nematoda, Onychophora (Grenier et al. 1997), Arthropoda, Nemertea (Kmita-Cunisse et al. 1998), Platyhelminthes (overview, Badets and Verneau 2009), Bryozoa (Passamaneck and Halanych 2004), Annelida (Bleidorn et al. 2009), and Mollusca (Degnan and Morse 1993). The nematode Caenorhabditis elegans has Hox genes but they are not clustered in its genome (review; Aboobaker and Blaxter 2003). This would indicate that the Hox cluster was secondarily dispersed in this lineage. The model arthropods Drosophila have Hox genes in clusters both complete and broken. For deuterostomes Hox genes have been found in the phyla Echinodermata (Dolecki et al. 1988), Hemichordata (Peterson 2004) and Chordata. For chordates the first Hox genes were discovered in the model organisms chicken, mouse and human (McGinnis et al. 1984b). They have also been found in more basal taxa including Urochordata and Cephalochordata (Bell et al. 1993; Holland et al. 1992).

Amphioxus (Branchiostoma floridae) is thought to contain the ancestral Hox gene cluster for the Gnathostomata (jawed vertebrates; Garcia-Fernandez and Holland 1994). Gnathostomata can be divided into 3 major classes: Chondrichthyes (cartilaginous fishes), Sarcopterygii (lobe-finned fishes) and Actinopterygii (ray-finned fishes). These classes all share the first instance of multiple (4) paralogous Hox gene clusters (Kim et al., 2000; Chiu et al., 2002; 2004; Prohaska et al., 2004; Raincrow et al. submitted). These are thought to have arisen via two rounds of genome duplication (Holland et al. 1994) but the cluster count of the intermediate species (lampreys and hagfish) is undetermined. The Hox genes in chordates are highly conserved and paralogs are easily discernible. This conservation has proven as useful for phylogenetics in chordates as it
has in determining membership in the protostome superphyla of Lophotrochozoa,
Ecdysozoa and Platyzoa. Central to the conservation of Hox genes in chordates is the conservation of their clustered formation. Clustering is yet one unique aspect of Hox genes.

### 1.2.3 Evolution of the Hox Gene Cluster

As mentioned previously, the first Hox gene clusters were discovered in Drosophila by E.B. Lewis (1978). Lewis first discovered what is now known as the BXC or bithorax cluster. The BX-C contains Hox genes ultrabithorax, abdominal-A and abdominal-B. The other Hox gene cluster found in Drosophila was known as the ANT-C or antennapedia cluster. The ANT-C was discovered by Kaufman et al. (1980) and contains Hox genes labial (Lab), proboscipedia (Pb), deformed (Dfd), sex combs reduced (Scr) and antennapedia (Ant). The first cluster of Hox genes in a vertebrate were found in mouse on chromsome 11 (Hart et al. 1985).

The most primitive animal with an intact Hox gene cluster found to date is the nemertean ribbonworm (Lineus sanguineus). This ribbonworm has a cluster containing 6 Hox genes (Kmita-Cunisse et al. 1998). Other more derived protostomes such as arthropods have evidence of both an intact (red flour beetle; Tribolium castaneum, Brown et al. 2002; Shippy et al. 2008, mosquito; Anopheles gambiae, Powers et al. 2000) as well as divided (D. melanogaster, silk moth; Bombyx mori, Yasukochi et al. 2004) Hox gene cluster, whereas more primitive protostomes such as nematodes (Burglin and Ruvkun 1993) and platyhelminths (Pierce et al. 2005) have dispersed Hox gene clustering.

The same variation in Hox gene clustering can be seen in deuterostomes but not to the same extent. The sea urchin (Strongylocentrotus purpuratus) has an intact Hox gene
cluster but the genes are not in the canonical order (Cameron et al. 2006). Amphioxus, a cephalochordate, contains an intact Hox gene cluster that contains 14 Hox genes that are all transcribed from the same strand (Garcia-Fernandez and Holland 1994; Ferrier et al. 2000). This is thought to be the archetypal state of Hox gene clusters for the chordates. After the divergence of Amphioxus and prior to the divergence of known gnathostomes it is postulated that two rounds of genome duplication occurred giving rise to 4 Hox gene clusters (Amores et al. 2004). For accounting purpose Scott (1993) developed a nomenclature system that assigns each of the four paralogous clusters a letter $(\mathrm{A}, \mathrm{B}, \mathrm{C}$, D) and orthologs to each of the 14 Hox genes found in Amphioxus 1-14. For example the gene orthologous to the $9^{\text {th }}$ gene on Amphioxus's Hox gene cluster will be known as HoxA9 on the A cluster, HoxB9 on the B cluster and so on. Each of these clusters is ordered the same as the Amphioxus Hox gene cluster but none of them contain the entire complement of 14 genes.

To date intact Hox gene clusters homologous to the 4 Hox gene clusters have been found in the cartilaginous fish horn shark (Heterodontus francisci, Kim et al. 2000), the lobe-finned fishes coelacanth (Latimeria menadoensis, Koh et al. 2003; Danke et al. 2004), Western clawed frog (Xenopus tropicalis), the domesticated chicken (Richardson et al. 2007), human (Acampora et al. 1989), mouse (Duboule and Dolle 1989), the domesticated dog (Canis familiaris), the ray-finned fish, Senegal bichir (Polypterus senegalus) and other species with whole genome sequences.

Several gnathostomes lineages have had further genome duplications or tetraploidization events but none of these occurred during a time that allows them to be both shared by many species and still be easily detectable. Over 27,000 species share the

FSGD and all species sequenced to date appear to have between 7-8 intact Hox gene clusters (Figure 3). For accounting purposes these Hox gene clusters follow the Scott (1993) nomenclature with 2 paralogous clusters per original gnathostome cluster (A > $\mathrm{Aa}, \mathrm{Ab} ; \mathrm{B}>\mathrm{Ba}, \mathrm{Bb} ; \mathrm{C}>\mathrm{Ca}, \mathrm{Cb} ; \mathrm{D}>\mathrm{Da}, \mathrm{Db})$. To date only fishes of the division Teleostei have been found to share the FSGD and it has been proposed that the FSGD is coincident with the base of the teleosts group although there is much evidence to the contrary. There are two main groups of teleosts based on numbers of species: superorder Ostariophysi (6,502 species) and superorder Acanthopterygii (13,421 species). Only a single fish from Ostariophysi has a complete genome and/or complete Hox gene cluster complement sequenced; zebrafish. Several species of Acanthopterygii have been sequenced including medaka (Oryzias latipes;Kurosawa et al. 1999; Naruse et al. 2004), 3-spine stickleback (Gasterosteus aculeatus; Hoegg et al. 2007), spotted green pufferfish (Jaillon et al. 2004), Japanese pufferfish (Kurosawa et al. 2006), Nile tilapia (Oreochromis niloticus; Santini and Bernardi 2005) and African cichlid (Astatotilapia burtoni; Hoegg et al. 2007). So far the two separate superorders can be differentiated based on their Hox gene cluster content as Ostariophysi has $\mathrm{Aa}, \mathrm{Ab}, \mathrm{Ba}, \mathrm{Bb}, \mathrm{Ca}, \mathbf{C b}$ and Da clusters and Acanthopterygii has Aa, Ab, Ba, Bb, Ca, Da and Db. This 8 Hox gene cluster architecture is considered the ultimate in Hox gene cluster evolution although the number of genes is only slightly higher than states prior (i.e. mouse 39 ; Tetraodon nigroviridis 48). Despite the slight increase in Hox gene number they are more tightly clustered and more modular in nature (Duboule 2007), which can lead to a greater flexibility of expression and co-option.

Known Hox gene cluster states throughout animal phylogeny leads to the question of what the ancestral state of the Hox gene cluster was and why the Hox gene cluster exists. There are 2 major theories on the ancestral state of the metazoan Hox gene cluster, but they both result in the following groupings. First, the Hox gene cluster is divided into 4 major categories: anterior, group 3, central and posterior genes. Anterior genes include group 1 and 2 of deuterostomes and Lb and Pb of protostomes. Group 3 genes appear to have an ancient origin and have been secondarily lost in protostomes, central genes include group 4-8 in deuterostomes and Dfd, Scr, Antp, Ubx and Abd-A in invertebrates and posterior genes include group 9-14 in deuterostomes and Abd-B in protostomes. The origin of each category is thought to lie in a single Hox gene. The origin of these 4 Hox genes is in question. There are competing theories that there were 2,3 or 4 original genes. For the 2 and 3 gene models the remaining genes were a result of tandem duplication of a more ancient Hox gene. A review of these models can be found in Garcia-Fernandez (2005).

The function of the Hox gene cluster has been a topic of debate. It is known that for species with an intact cluster that they show spatiotemporal colinearity with their expression along the anterior-posterior axis of the developing embryo (McGinnis and Krumlauf, 1992; Lufkin, 1996). For this reason it was postulated that the positioning of the Hox genes along the cluster and their tight cluster formation, which excludes any other genes, was necessary (although with an unknown mechanism) for proper spatiotemporal patterning. This is not true however as found for animals that do not have intact Hox gene clusters yet still maintain spatial patterning such as the fruit fly, the nematode and a tunicate (Seo et al. 2004; review in Ferrier and Minguillon 2003). As
described by Monteiro and Ferrier (2006), these species all share rapid development that does not lend itself to the temporal aspect of gene induction. This still allows clustering to be necessary for temporal patterning however. Chambeyron and Bickmore (2004) hypothesized that there could be a structural component to the cluster where the orientation and proximity were needed for proper expression pattern. They were able to show that the anterior portion of the HoxB cluster in mice (HoxB1-9) underwent chromatin decondensation in response to a known Hox gene inducer, retinoic acid. Then in sync with their expression pattern each gene "looped out" from the chromatin structure towards the center of the nucleus. It is possible that the pressures necessary to maintain the cluster formation are different in different lineages and we may never know what the original pressure was. The loss of the clustering formation in some species indicates that it is not essential for bilateral life but its widespread occurrence at least indicates that it is extremely advantageous.

The widespread occurrence of Hox genes, their clustered nature and role in the formation of the body plan make them an ideal candidate to study differences and similarities between wide-ranging species and, as we will show in chapter 4 , between closely related species. This is especially true in teleosts since many Hox genes have been maintained as duplicates and since accelerated and asymmetric rates of nucleotide evolution are thought to be one molecular contribution to the success of this clade.

## Chapter 2- Closing the Gap between Teleosts and Non-Actinopterygians

### 2.1 Introduction

### 2.1.1 Ray-finned Fish Phylogeny

Comparative genetics has been widely used to help decipher the function of the vast amounts of non-coding DNA in the genome (Lee et al. 2006; Siegel et al. 2007; Sato et al. 2009). In gnathostomes, the predilection towards humans and 'model' organisms has resulted in conducting the majority of long-range comparative genomics between human, mouse, chick (Sarcopterygians), zebrafish, pufferfishes (Actinopterygians) and sometimes sharks (Chondricthyians). While studies of this nature are useful at finding patterns of conservation/loss, it is also important to understand how these patterns arise. To do this a wider taxonomic sample of each order is necessary. In Sarcopterygians the inclusion of reptiles and frogs and possibly even lungfishes and coelacanths will be useful. In Actinopterygians however the knowledge of more basal taxa is limited and therefore we will give a short review.

Actinopterygii (the ray-finned fishes) is subdivided into 4 major clades Polypteriformes (bichir and reedfish), Chondrostei (sturgeons and paddlefish), Holostei (gar and bowfin) and the crown group Teleostei (all remaining ray-finned fishes) (Nelson 1969). Compared to cartilaginous and lobe-finned fishes, the ray-finned fishes have undergone a remarkable number of whole genome duplications, which makes genome comparisons slightly more complicated. The teleosts are by far the most successful extant ray-finned fish clade with 38 orders, 426 families, 4,064 genera and $>27,000$ species (Nelson 2006). All teleosts share a whole genome duplication dubbed the FSGD (Jaillon et al. 2004). One hallmark of the teleost clade is additional rounds of whole
genome duplications in a lineage-specific manner, e.g. salmonids (Ohno 1970; Allendorf and Thorgaard 1984) which have tetraploid genomes and as many as sixteen Hox clusters (Moghadam et al. 2005a; Moghadam et al. 2005b). Interestingly, evidence suggests the evolution of basal ray-finned fishes like gar (Ohno 1969) and paddlefish (Dingerkus and Howell 1976) may also be associated with lineage-specific duplication events.

As shown in Figure 2, Amiiformes, represented by a single extant species called the bowfin (Amia calva) is the closest sister group to the crown group of teleosts (Nelson 1994; Bemis et al. 1997). The bowfin lives in eastern North America and there is no indication of polyploidy in this species.

The next closest order is Semionotiformes (Nelson 1994; Bemis et al. 1997)
(Figure 2). Semionotiformes are comprised of two extant genera with seven species known commonly as gars. Gars can be found in eastern North America and Central America. It has been postulated that gars may have undergone their own genome duplication (Ohno et al. 1969; Dingerkus and Howell 1976).

Order:Acipenseriformes (Figure 2) contains two families (Acipenseridae,
Polypdontidae; Nelson 1994) comprised of six genera and twenty-six species known commonly as sturgeons and paddlefish. The twenty-four living species of sturgeons are found throughout the Northern Hemisphere; the two living species of paddlefish are found in the United States and China. Sturgeons have undergone at least three wholegenome duplications that result in phylogeny that can be deduced via chromosome number (Fontana and Colombo 1974; Ludwig et al. 2001; Fontana et al. 2008). Paddlefish appear to have undergone at least 2 whole-genome duplications (Dingerkus and Howell 1976).

The most basal ray-finned fish clade is that of the order Polypteriformes. Commonly known as bichirs and reedfish, Polypteriformes contains two genera and ten species. Evidence suggests that bichir has not undergone its own genome duplication (Chiu et al. 2004; Mulley et al. 2006; this study). Strikingly, the stem ray-finned fishes comprise $\sim$ forty-four species or less than $1 \%$ of all ray-finned fishes. Despite a handful of useful candidate species among the basal ray-finned fishes (such as bichir and bowfin), most authors focus only on comparisons between outgroups to the Actinopterygians (Sarcopterygians; Chondrichthyians) and teleosts. We will present evidence that suggest many patterns and possible hypotheses are missed due to this simplistic approach.

### 2.1.2 Teleost Bias

The majority of gen(om)e comparison studies that have been conducted comparing Sarcopterygians and/or Chondrichthyians to Actinopterygians have focused on members of the teleost clade (Figure 2). Siegel et al. 2007 compared the ParaHox clusters of several teleost fishes to those of human and mouse. In this study they find that teleosts do share some conserved gene order and conserved non-coding sequences with mammals. They also found several non-coding DNAs that are conserved only among teleost fishes. But without more basal Actinopterygii in the analysis, there is no way of knowing if these CNS are novel to teleosts or are the result of cis-regulatory sequences that evolved in ray-finned fish stem lineages. The lack of phylogenetic depth in this study skews their conclusions about how the ParaHox clusters have evolved in the teleosts. This same type of problem arises in phylogenetic comparative work done by other authors as well (Lee et al. 2006).

Sato et al. 2009 conducted loss/retention analysis of 130 genes of taste and olfactory transduction and tricarboxylic acid cycle pathways. Again they compared several teleosts genomes to that of human and determined that one-half of the genes were present prior to the FSGD and $40 \%$ of those were maintained as duplicates. Many more genes may have been present prior to the FSGD and subsequently lost after the FSGD although this is unknown given that there is $\sim 150$ my of evolution missing in the comparison. One may conclude that this missing information is not relevant given that the authors want to compare teleosts to any state prior to their genome duplication. However, without information of how ray-finned fishes evolved prior to the FSGD one has no way of knowing how or why genes are retained or lost after the FSGD and how this has contributed to the evolution of the teleosts. Although the data can stand on its own as far as lost/retention rates, the conclusions drawn from this data are lacking a certain understanding of immediately prior states. This point is argued in Johnston et al. (2007) where the authors find that genes are more likely to be maintained as duplicates if they were undergoing accelerated evolution prior to the duplication.

Another assumption that is made using this type of data is that teleosts have lost several DNA regions including genes and CNSs that are shared between lobe-finned and cartilaginous fishes (Chiu et al. 2002; Venkatesh et al. 2007; Yu et al. 2008). This loss is often contributed to an accelerated mutation rate and/or the FSGD (which may or may not be mutually exclusive events). However, when stem-ray finned fish data is included in these types of studies one finds that this turnover started prior to the teleost radiation (Chiu et al. 2004; this study). Ravi and Venkatesh (2008) addresses this issue as well but still fails to use any stem-ray finned fishes in their study.

The lack of data from pre-FSGD stem ray-finned fishes is glaring in phylogenetic studies. For example, a recent study attempted to use EST data to help solve gnathostome phylogenomics for the major clades (Chondrichthyes, Sacropterygii, Actinopterygii and Tetrapoda; Hallström and Janke 2008). They contributed lungfish (Protopterus aethiopicus) DNA sequence to the study in order to expand the depth of Sarcopterygian sampling, but despite having 4 teleosts, included no stem ray-finned fishes. It is impossible to confidently arrange these major clades without data from preFSGD ray-finned fishes. Similar work was done by Martin (2001).

If pre-FSGD stem ray-finned fishes are needed for genome comparison, then which are ideal candidates? Chiu et al. (2004) used bichir, the most basal, extant rayfinned fish. We propose that this is an ideal pre-FSGD ray-finned fish given its place in phylogeny as well as its apparent lack of a lineage specific genome duplication. Comparisons using bichir will add data from a genome that should be similar to Chondricthians and Sarcopterygians structurally, but undergone selective pressures of an Actinopterygian.

### 2.1.3 Bichir Phylogeny and Anatomy

At the base of the Actinopterygian tree is the genus Polypterus (bichir). Despite this phylogenetic position, bichir is not frequently used in studies. Perhaps this is because only recently has bichir held that phylogenetic position.

Polypterus is an enigmatic taxon with many shared and unique features that have given taxonomists constant headaches (Figure 4). Among the shared features are: 1 . Lobe-like fins (Sarcopterygii) 2. Ganoid scales (Sarcopterygii) 3. 2 gular plates (Coelacanthimorpha:Sarcopterygii and Holostei) 4. Spiracles (Chondrostei) 5.

Feathery external gills and double ventral lungs as juveniles (Dipnoi:Sarcopterygi) and 6. Heterocercal tail (Chondrostei) (Janvier 1996).

Among the unique features are: 1 . Dorsal fin rays that project horizontally from a vertical spine (Helfman et al. 1997) 2. Recoil aspiration (Brainerd et al. 1989) 3. Heterocercal tail, external gills and lobe-like pectoral fins are due to homoplasy and are anatomically different (Janvier 1996). Early systematists had difficulty classifying bichir using anatomical features and they were placed in at least four separate clades including the Dipnoi (lungfishes), Ganoidei, three separate groups of Actinopterygii (Teleostei, Holostei, and Cladistia) and an entirely separate subclass known as Brachiopterygii which was held in equal weight with the Actinopterygii and Sarcopterygii (Helfman et al. 1997). A consensus was reached in 1982 when Patterson placed Polypteriformes as the basal group to the Actinopterygii. After the advent of molecular phylogeny much work has been done and today it is accepted that bichir is indeed the most basal rayfinned fish (Inoue et al. 2003; Kikugawa et al. 2004; Figure 2).

Due to the diligent work put into phylogenetically positioning bichir among other fishes we can now use bichir in studies requiring a basal ray-finned fish. But without knowing the ploidy level of bichir we may still incur the issues that arise with other stem ray-finned fishes like the sturgeons, paddlefish and gars. For this reason our lab has put in great effort to help determine whether or not bichir has undergone a lineage specific genome duplication or at the very least chromosomal duplication resulting in greater than 4 Hox gene clusters.

### 2.2 Bichir Hox Gene Discovery

### 2.2.1 PCR Discovery and Sequencing of Hox Genes

## Materials and Methods

Degenerate primers were designed from alignments of vertebrate Hox genes for HoxC4 and HoxD9 (C4 Forward 5'-ATG ACG TCG TAT TTG ATG-3'; C4 Reverse 5'TGA TTT GCC TCT CGG AG-3'; D9 Forward 5’-GTA TTG GTA AAT ATG ATC ACG-3'; D9 Reverse 5'-CKG TTC TGA AAC CAG ATT TT-3'). PCR reactions were setup using reagents from Applied Biosystems ${ }^{\mathrm{TM}}$ AmpliTaq ${ }^{\circledR}$ DNA Polymerase kit ( N 8080153 ) in the following concentrations: 1 X buffer, $3.5 \mathrm{mM} \mathrm{MgCl} 2,0.8 \mathrm{mM} \mathrm{dNTP}$, and 0.5 uM primers. 50 ul reactions were made with 3 ul genomic DNA (unknown concentration), 2.5 units Taq DNA polymerase and diluted with sterile water. Negative controls were made under identical conditions except genomic DNA was not added. The PCR was run using a MJ Research PTC-200 DNA Engine thermalcycler under the following conditions: 95 C for 5 minutes, 30 cycles of ( 95 C for 1 minute, 47 C (HoxC4) or 52 C (HoxD9) for 1 minute, 72 C for 2 minutes), 72 C for 10 minutes. 5 ul of each PCR reaction was mixed with 1 ul 6 X loading dye and loaded onto a $2 \%$ agarose gel (1X TAE) along with 4 ul of 1X Invitrogen 1kb DNA ladder (15615-024). Gels were run on an Owl brand electrophoresis unit at 60 milliwatts for 1.5 hours. Bands of correct size were excised from the gel and purified using Qiagen Gel Extraction Kit (28706) under standard protocol except DNA was eluted using 10 ul sterile water. Gel purified DNA was cloned into Promega pGEM®-T Vector System II (A3610) using standard protocol except reaction was incubated at 16 C for 20 hours. 2 ul of ligation reaction was transformed into 25 ul Escherichia coli Promega JM109 Competent Cells (L2001) under
the following protocol: on ice for 20 minutes, 45 C (water bath) for 42 seconds, on ice for 2 minutes, add 240 ul LB broth, 37 C for 1 hour at 200 r.p.m. The transformed cells were plated on to LB agar plates containing $50 \mathrm{mg} / \mathrm{mL}$ ampicillin, 1M IPTG and X-gal and grown at 37 C for 16 hours. Colony PCR was performed on white colonies under identical conditions as original PCR except in 20 ul reactions. Colonies with band of correct size were grown in 3 mL of LB broth at 37 C for 16 hours at 200 r.p.m. Plasmids containing insert of correct size were purified using Qiagen QIAprep Miniprep Kit (27106) using standard protocol except DNA was eluted using 30 ul sterile water. 300 ng of plasmid DNA was sent for sequencing using the T7 primer to the DNA core facility at the University of Medicine and Dentistry of New Jersey, Pisctaway, NJ.

Chromatograms of the DNA sequences for HoxC4 and HoxD9 were analyzed for errors. Resulting DNA sequences were then trimmed of vector and primer and blasted against the non-redundant database at http://blast.ncbi.nlm.nih.gov/. Verification of Hox paralog identity was based on identity of top matches from this blast. After verification of paralog identity, sequences were analyzed via phylogenetic inference. Alignments were created of all known paralogs using ClustalW algorithm provided by MacVector® 9.5.2. The alignment was corrected by eye so that all sequences started at the same position. Neighbor-joining, Maximum Parsimony, Maximum Likelihood and Bayesian trees were created from these alignments. Neighbor-Joining and Maximum Parsimony trees were created using default parameters in PAUP* v4.0b10 (Swofford 2003). Maximum Likelihood trees were created using GARLI v0. 951 (Zwickl 2006), which can be downloaded from www.bio.utexas.edu/faculty/antisense/garli/Garli.html. The starting tree was obtained using heuristic search under the likelihood optimality criterion in

PAUP* v4.0b10 (Swofford 2003) with settings as specified by Modeltest (Posada and Crandall 1998, see Model Selection). The same settings were inputted into GARLI v0. 951 (Zwickl 2006). Node confidence was assessed by using the bootstrap resampling method with 2,000 replications. The "number of generations without improving topology" setting was changed from 10,000 to 5,000 as suggested in the GARLI manual when doing bootstraps, as it will "shorten the run time without significantly affecting the results". A consensus tree was created using majority rule with a cutoff of $50 \%$ in PAUP* v4.0b10 (Swofford 2003). Bayesian trees for the nucleotide alignments were constructed using MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003) and the parallel version of MrBayes v3.1.2 (Altekar et al. 2004) under the settings specified by MrModeltest. Two independent Markov Chain Monte Carlo analyses were run with the following settings: number of generations was set to $1,000,000$, sample frequency was taken every 1,000 steps, number of chains was set to 4 and the temperature was set 0.2 . 'Burnin' was assessed after the run using the sum parameters command. The 'burnin' for the nucleotide analysis was set to 1 for all 3 datasets, which is equal to the first 1,000 steps or tree topologies. A majority rule consensus tree was created disregarding the 'burnin' trees using the sum trees command with a cut off of 0.50 posterior probability.

### 2.2.2 BAC Library Screening

## Materials and Methods

A 5-fold coverage bichir genomic bacterial artificial chromosome (BAC) library (Chiu et al. 2004) was screened for presence of bichir Hox genes using a DNA probe of Hox C 4 and HoxD9. High-density 5 X 5 arrayed filters were made by the RZPD (http://www.rzpd.de). Southern hybridization was performed on these filters. DNA
probes were labeled with a non-radioactive marker, digoxigenin. We DIG (Digoxigenin)labeled the HoxC4 and HoxD9 fragment using Roche Diagnostics DIG DNA labeling kit (11175033910). The DIG labeling reaction includes boiling $800 \mathrm{ng}(15 \mathrm{ul})$ of the HoxC4 or HoxD9 fragment in water for 10 ', placing on ice $\left(\mathrm{H}_{2} \mathrm{O}\right)$ for 10 ', briefly spinning down, then adding 2 ul of the hexanucleotides, 2ul dNTP labeling mix, and 1ul Klenow enzyme provided in Roche kit. We then placed the labeling reaction at 37 C for 20 hrs . Southern hybridization was performed on the 5 X 5 filters in a Lab-Line rotisserie incubator at 65 C. Briefly, filters were "pre-hybridized" at 65 C for 5 hrs . DIG-labeled probe was added to pre-hybridization solution at a concentration of $1 \mathrm{ul} / \mathrm{ml}$. Pre-hybridization solution was then replaced with hybridization solution and incubated for 20 hours at 65 C . Filters were then washed with the following steps all at room temperature: rinsed in 2X SSC ( 300 mM sodium chloride, 30 mM sodium citrate), washed in first hybridization wash (2X SSC, $0.1 \% \mathrm{v} / v$ SDS-sodium dodecyl sulphate) for 20 minutes while rocking, washed in second hybridization wash (1X SSC, $0.1 \%$ SDS) for 20 minutes while rocking, rinsed in diluent buffer ( $300 \mathrm{mM} \mathrm{NaCl}, 100 \mathrm{mM}$ Tris ph 8.0 ) for 20 minutes while rocking. Filters were then saturated by $1 \%$ blocking solution (Roche 11096176001) for 1 hour while rocking. Alkaline phosphatase conjugated antibody to DIG (Roche 11093274910) was applied to filters in a $1 / 1000$ ratio in $1 \%$ blocking buffer for 30 minutes while rocking. Filters were once again washed in a detergent solution $(0.3 \% ~ v / v$ Tween in diluent buffer) 3 successive times for 10 minutes while rocking. Filters were then incubated in detection buffer ( 1 mM Tris $\mathrm{pH} 9.5,0.1 \mathrm{M} \mathrm{NaCl}$ ) for 5 minutes. Filters were then affixed to old film panel and CDP star substrate (Roche 12041677001) was applied and covered with thin sheet of poly vinyl chloride. The substrate reacts with the alkaline
phosphatase to emit light, which is detected by film in a dark room. Coordinates of positive clones are determined in duplicate on a 2 clone per array basis.

A group of 4 clones were discovered with 1 or both HoxC4 and HoxD9. Due to the conflicting data from these hybridization experiments the bichir BAC library was screened via PCR for presence of Hox C 4 containing clones using bichir specific primers (PseC4forward 5'-ATG AGG TCG TAT TTG ATG-3' and PseC4reverse 5'-TGA TTT GCC TCT CGG AG-3').

The bichir BAC library consists of 216 pools each of which contains 384 clones. The PCR reactions were setup similar to that of the genomic DNA isolation except they were scaled to 16 ul. The same PCR program was performed as for the genomic DNA isolation. No pool contained a single distinct band corresponding to the expected product therefore this analysis was not extended.

Earlier, bichir BAC clone CLN84 (AC135508) was isolated that contained the HoxD1 gene. This BAC clone was sent to Eric Lander at the Whitehead Institute/MIT Center for Genome Research to be sequenced. Annotation of the clone was performed to yield 3 Hox genes; HoxD1,HoxD2 and HoxD3.HoxD2 is the first D2 paralog that has been found among bony-fishes and supports the idea that bichir may represent a basal ray-finned fish. Phylogenetic analysis clearly defines HoxD1, HoxD2 and HoxD3 as 'D' paralogs of their specific Hox groups (Figure 7). There is no evidence of a HoxD4 gene upstream of HoxD3 despite 20 kb of sequence. The HoxD3 gene has the largest known intron of any vertebrate Hox gene. The HoxD3 intron is $16,255 \mathrm{bp}$ and contains one intact Tc1 DNA transposon as well as remnants of Rex like non-LTR retrotransposons (see below). Bichir BAC clone M919 (AC138742) was annotated and found to contain 2
of the 3 exons of EVX2 (Figure 7) a vertebrate ortholog of the Hox domain containing gene in D. melanogaster, even-skipped. A HoxB containing bichir BAC clone (AC138147) was also found. This BAC clone contains the entire open reading frame (ORF) of HoxB5, HoxB8, HoxB10, HoxB13 and partial sequence for HoxB4 and HoxB7 (Figure 7). The partial sequences are most likely due to incomplete sequencing of the BAC clone and not due to a pseudogene status for HoxB4 and HoxB7.

### 2.3 Hox Gene Data Suggest Bichir is a Basal Ray-finned Fish

Phylogenetic results indicate that HoxC4 and HoxD9 of bichir were assigned to the correct clusters and paralog groups and each supports bichir's assignment as the most basal living ray-finned fish (Figure 5). Previous work has suggested that bichir does not have its own specific genome duplication (Chiu et al. 2004; Mulley et al. 2006), which is seen in other basal ray-finned fishes such as paddlefish, sturgeon, and gar (Ohno et al. 1969; Fontana and Colombo 1974; Dingerkus and Howell 1976; Ludwig et al. 2001; Fontana et al. 2008). Hox C 4 is the first discovery of a HoxC paralog gene in bichir. HoxD9 adds to the annotation of HoxD1,HoxD2,HoxD3,HoxD12 and EVX2 in bichir that were found via clone identification and sequencing.

The discovery of several linked HoxB and D cluster genes as well as a HoxC cluster gene has helped verify that bichir has not undergone its own genome duplication (in agreement with Chiu et al. 2004 based on the HoxA cluster) and has also lent information to the evolution of Hox gene clusters in vertebrates as bichir has the first instance of a group 2 gene on the HoxD gene cluster in Osteichthyes and also the first instance of a group 1 gene on the HoxD gene cluster in Actinopterygii (Figure 6).

### 2.4 Conclusion

Bichir is in an important phylogenetic position among ray-finned fishes. Diverging from the rest of the ray-finned fishes prior to the FSGD is just one important factor. Bichir also has the distinction of being the most basal living ray-finned fish and according to the data gathered during this and previous work (Chiu et al. 2005; Mulley et al. 2006) does not appear to have undergone a separate lineage-specific genome duplication. Because of these factors it is important and advantageous to include bichir in all analyses determining the effects, or lack there of, of the FSGD on the evolution of ray-finned fishes. As will be presented in the following chapter, data from bichir Hox genes and Hox gene clusters has lent valuable information to the evolution of Hox gene clusters prior to the FSGD that supports the notion the duplication alone is not sufficient in explaining the genomic and phenotypic plasticity seen among the teleost lineage of ray-finned fishes.

## Chapter 3- Re-analyzing Teleost Uniqueness

### 3.1 Introduction

### 3.1.1 Cis-regulatory Elements

In this chapter we present an argument of why cis-regulatory elements are important to evolution, how cis-regulatory elements have evolved in the conserved Hox gene clusters and how the addition of bichir affects the analyses of the changes observed in the non-coding DNA of teleosts Hox gene clusters.

We will start with a background of cis-regulatory elements, their detection and their importance. The majority if not all eukaryotic genes contain expression level controlling DNA sequences called cis-regulatory elements (CRE). These elements are normally found flanking the transcribed portion of the gene but may also be found within introns and even exons (including the coding region). CREs are supplementary to the promoter sequence that is responsible for binding RNA polymerase and its associated proteins.

The first CRE described was the lac operon (Jacob and Monod 1961). This operon is responsible for controlling the response to the presence or absence of glucose/galactose in the environment. From this knowledge came the idea that CREs are switches that could turn on or off the production of a protein. We now know that regulation can occur at many levels from DNA to protein. We also know that CREs are not simply 'on/off’ switches. CREs may also work more like a dimmer switch controlling not only if RNA will be made but also how likely and how often RNA will be made. This is possible because CREs are made up of a bundle of transcription factor binding sites (TFBS) that bind proteins responsible for either enhancing or suppressing
transcription. CREs respond specifically to the transcription factor environment allowing them to regulate their genes in a context dependent manner, in many instances at specific time-points during ontogeny and in specific tissues.

Complimentary to their role in development and tissue maintenance, CREs are likely to be targets of natural selection at the population level (see 3.1.4) and hence play an important role in genomic (Vavouri and Lehner 2009) and phenotypic evolution (Gompel et al. 2005). Despite their importance, little is known about CREs. This is due to the difficulty in identifying CREs in the genome because they do not code for a specific gene product and may be located far from the gene(s) whose action they control. Multiple methods have been developed to identify CREs. First we will look at traditional wet lab approaches and then computer-based approaches derived from them.

### 3.1.2 Traditional Approaches to Locating Cis-regulatory Elements

Traditional approaches to finding CREs include DNase footprinting, electromobility shift assays (EMSA) and deletion analysis, which can all be followed by transgenics. The first approach, DNase footprinting, consists of binding specific transcription factors (TFs) to a known sequence and digesting away the exposed, singlestranded DNA leaving behind only the DNA protected by the TF; termed the TFBS (transcription factor binding site), Using this approach, Galas and Schmitz (1978) first reported that TFBS are often clustered in genomes, which is one important trait of CREs.

The second approach, EMSA, involves incubating a specific DNA sequence with either proteins hypothesized to bind to the sequence or whole nuclear protein extracts and identifying DNA-protein complexes by their retarded rate of migration in gels, or 'shift'. While the EMSA procedure alone cannot identify the specific protein that is bound to the
known DNA sequence, expansion of this method (supershift; Kristie and Roizman 1986) has proven powerful in identifying CREs of known TFs.

Using these traditional wet lab approaches many TFBS have been identified. Several databases such as TRANSFAC (Matys et al. 2003) and JASPAR (Sandelin et al. 2004) store all these known TFBS and allow one to submit an unknown DNA sequence thought to contain TFBS and have a search algorithm identify any similarities. The search algorithm works by creating a degenerate binding matrix for a transcription factor with known binding sites and then aligning that to the sequence in question. The degenerate binding matrix is designed by giving scores to the presence of one of the four nucleotides $(\mathrm{A}, \mathrm{C}, \mathrm{G}, \mathrm{T})$ at each position relative to the frequency they appear in known binding sites. Sometimes certain nucleotide positions are weighted heavier overall (core sequence) due to their inclusion in most if not all TFBS for their particular TF. These programs can do this for every transcription factor in their database so as to provide one with as complete of an array of possible TFBS for their sequence as possible.

The third approach, deletion analysis, has been used to detect cis-regulatory elements for many genes including Hox genes (Shashikant et al. 1995; Nonchev et al. 1996; Maconochie et al. 1999). Deletion analysis involves removing portions of the DNA upstream of a gene and looking for indication of loss of expression. After expression is lost the region deleted can be narrowed for higher resolution. All of these methods are, of course, indirect evidence of cis-regulatory activity and can be supplemented by transgenics.

Transgenics uses reporter gene assays, which involve placing a putative cisregulatory upstream of a minimal promoter and a marker gene (coding for a visible
protein; ie. green fluorescent protein or a protein that can be secondarily visualized; ie. $\beta$ galactosidase). This construct is then transiently or permanently transfected into a model organism. Via transfection one can test for recapitulation of the endogenous expression pattern of the gene thought to be regulated by the putative cis-regulatory element in question and/or identify other possible regulated genes.

### 3.1.3 Comparative Genomic Approaches to Locating Cis-regulatory

## Elements

In the post-genomic era, comparative genomics has become a powerful approach to identifying CREs. In this method, orthologous non-coding DNA sequences from two to several groups of organisms, which collectively add up to enough evolutionary time that similarities in their sequences can be considered not likely by chance, are aligned. This approach is based on the observation that CREs evolve at a slower rate than surrounding non-coding DNA, presumably because they have a function(s) and are subject to purifying selection (Keightley et al. 2005). Evolutionarily conserved noncoding sequences (CNS; Hardison 2000) are putative CREs. In this approach, the depth of evolutionary time necessary for this analysis differs based on parameters including relative mutation rate, genome size, and generation time (review, Martin and Palumbi 1993). Protocols implementing this method include rVista (Loots and Ovcharenko 2004), TRACKER (Prohaska et al. 2004), and MONKEY (Moses et al. 2004).

Phylogenetic Footprint Clusters (PFCs) are a stringently defined type of CNS with specific methods of detection. PFCs are defined as CNSs that between any two species contain 2 or more "footprints" within 100 nucleotides of each other (Chiu et al.
2002). A footprint is defined as a string of at least 6 nucleotides that are conserved $100 \%$ between two or more species being compared that, for mammalian lineages, represent at least 250 million years of additive evolutionary time (Tagle et al. 1988). This method was modeled after DNAse footprinting (see 3.1.2; Galas and Schmitz 1978).

Evolutionary biologists determine putative TFBS by allowing evolution to do the "digestion" that can be seen when comparing homologous regions of two distantly related organisms whereby there has been enough evolutionary time that the surrounding nonbinding sites have diverged and only the TFBS are still conserved (Tagle et al. 1988).

Phylogenetic footprinting has shown to be a useful method in the search for CREs. The majority of known CREs in Hox clusters can be found by this method, although, the amount of false positives has not been assessed due to the amount of time necessary to empirically test each PFC that does not already correspond to a known CRE. A system such as the Hox clusters lends itself well to this type of CRE inquiries for several reasons. 1. Identifying orthologous non-coding DNA is highly accurate due to the highly conserved nature of the cluster formation. 2. Because of the importance Hox genes have to development, their expression patterns have been well researched and documented. 3. Despite this seeming understanding of the system very few Hox genes have had their CREs identified in much detail.

### 3.1.4 Cis-regulatory Elements vs. Transcription Factors

Cis-regulatory element mutations are thought to be responsible for the majority of novelty needed for adaptation in comparison to coding sequence mutations. This hypothesis was first put forth based on the structure and function of CRE vs. coding sequence (Monod and Jacob 1961; Britten and Davidson 1971). Cis-regulatory elements
are modular in nature and hence may easily overcome the problem of pleiotropy that acts against changes in protein coding sequences. Cis-regulatory elements can drive the expression of a particular gene in many different stages of development as well as in many different tissue types. Depending on which TFs are being expressed in any particular cell or at a given time (developmental, seasonal, etc.), the CRE can respond in kind to either suppress or enhance transcription of the gene it regulates. Because of this cell environmental plasticity, it is possible to change the expression of the gene in one cellular context without affecting the expression in another cellular context (reduced pleiotropy). Coding sequences on the other hand incur mutations that $69 \%$ (148/216) of the time change the amino acid sequence that it codes for and thus the protein that it produces.

It is thought that changes in the amino acid sequence would be highly pleiotropic in that a change to the protein structure would affect the function of the protein in all contexts (of course this can be overcome somewhat by alternative splicing and domain specific contexts). Based on these ideas it is proposed that CRE evolution can play a large role in adaptation. There are many experiments to show that this is indeed the case; well-known examples include pigmentation in the fruit fly (Wittkopp et al. 2002; Gompel et al. 2005) and pelvic reduction in sticklebacks (Shapiro et al. 2004). At the population level, CREs have shown to play a role in the adaptation of malaria resistance (Tournamille et al. 1995), lactase persistence (Bersaglieri et al. 2004), and blood clotting in humans (Rockman et al. 2005).

This is an important distinction because all evolution must take place at the population level where changes in allele frequencies cause changes in the species
phenotype as a whole. That CREs would be a more likely source of functional variation/adaptation at the population level is not surprising. As summarized by Wray (2007), expression differences in alleles are independent which means that CREs controlling the expression are co-dominant, whereas coding sequence mutations are more often than not recessive. This is important because for a beneficial allele to reach a frequency in a population that is measurable and effectual it must be selected for in the heterozygote. This is the case because before a sufficient number of homozygotes appear in the population the allele will likely be lost to drift.

There are many good arguments for why CREs play a larger role in adaptation and it appears at first glance that the empirical evidence agrees. But, it is also important to assess the assumption that is made to reach this hypothesis; changes to the amino acid sequence will have pleiotropic effects. This is not necessarily the case. Alternative splicing is one such form that may overcome pleiotropy. Also, proteins have many domains especially transcription factors. Transcription factors in particular have a DNA binding domain that should stay well conserved, changes in this region will almost surely be pleiotropic. But, transcription factors also have protein-protein interaction domains that help them bind to other proteins that are apart of the enhancer/suppressor complex that regulates transcription for the gene they are helping to control. Changes in these regions may or may not be completely pleiotropic such that a transcription factor will not always be binding with the same protein partners in different context.

Lending evidence to this type of modularity are motifs known as Short Linear Motifs (SLiMs) that help regulate these protein-protein interactions (Neduva and Russel 2005). SLiMs are normally 6-10 amino acids in length and often can be modular. Re-
arrangements of the amino acids can occur giving them a different binding profile and only a few changes can completely inactive or cause a de novo binding. In this way, SLiMs can overcome the pleiotropic effects that plague proteins coding mutations and be selected for very much the same way as a CRE.

Despite the possibility of a protein to overcome pleiotropy there is very little evidence that this occurs. There may be other factors that are not considered that keep proteins from overcoming pleiotropy. One reason may have to do with the difficulty to select for these methods at the population level. Which as is pointed out earlier in this paper is necessary to become a contributing allotype. SLiMs may be able to overcome this evolutionary snag but to date very little is known about them and there is no such evidence to support that they do indeed overcome pleiotropy. So until SLiMs or another method is better classified it is still the consensus that the majority of selection on mutations that affect development should occur in CREs.

### 3.1.5 Cis-regulatory Elements and Hox Gene Cluster Evolution

CREs are important to the evolution of Hox gene clusters. CREs are the most likely targets of natural selection, especially for higher order regulatory genes such as Hox genes. This is apparent in how the Hox genes accomplish their role. The function of Hox genes is to bind to their downstream targets (CREs) and either enhance or suppress the expression of the genes these CREs control. The main result of this transcriptional regulation is locating in space and time the formation of particular structures along an axis. Hox genes accomplish this spatiotemporal specificity via overlapping expression profiles setting up specific domains during development of the embryo. For this reason, changing the Hox gene's own spatiotemporal expression is the most effective way to alter
their function. This is accomplished by altering the CREs of the Hox genes themselves thus changing their ability to respond to particular sets of upstream TFs. The result of this alteration will be the Hox gene and all of its downstream effectors being expressed in a different location in the embryo and thus altering the positioning along the axis.

The importance of the CREs of Hox genes can also be seen via work in the McGinnis lab (McGinnis et al. 1990; Malicki et al 1993) which has shown swapping out the coding region of Hox genes between Drosophila and mammals still allows them to function normally because they are being expressed at the right time and location. The importance of CREs also comes up in the discussion of the maintenance of the cluster formation. Several evolutionary biologists (Peifer et al. 1987; Kmita and Duboule 2003; Santini et al. 2003) have postulated that one reason the Hox genes maintain a clustered formation is because they locally share CREs (Gould et al. 1997). The question of whether or not or how much of a role local CRE sharing plays in maintaining the cluster formation is still open to debate and it is important to understand the maintenance pattern of CREs to determine the likelihood of this hypothesis.

### 3.2 Hox cluster Conserved Non-coding Sequence Database

### 3.2.1 Need for Comprehensive Database

Previous attempts have been made to catalog the CNSs within the Hox gene clusters of gnathostomes (Chiu et al. 2002; Santini et al. 2003; Chiu et al. 2004). These attempts were made in an effort to understand the relationship between species that contain 4 Hox gene clusters and those that contain more. In these studies a representative species is chosen from 2 or 3 clades of gnathostomes and then their Hox gene clusters are aligned and scanned for CNSs. The issue with these comparisons, which we hope to resolve, was the exclusion of species due to lack of sequencing data or the desire for simplicity.

As described in Chiu et al. (2004) it is important to include basal ray-finned fishes. Also apparent in this study is the knowledge that, at least for the teleost fishes, species thought to be similar may have a quite distinct retention patterns with the same disparately related species. For this reason we have attempted to create a comprehensive database of CNSs for all available gnathostome Hox gene clusters in an attempt to better understand the pattern of retention and loss of CNSs in these otherwise highly conserved gene clusters. Understanding this pattern will lead to a better understanding of the forces that act to resolve (or not) redundancies in conserved genetic systems that undergo replication. We also hope that establishing a comprehensive database with an informative and flexible naming system will lead to better communication between labs and help expedite searches for both the function of these CNSs as well as their contribution to the cluster formation of the genes they regulate.

### 3.2.2 Annotating Hox clusters

## Materials and Methods

The Hox clusters used in this study were constructed by a variety of methods. Homo sapiens (human) HoxA cluster was taken from a shorter fragment of a contig made from clones with accession numbers AC004079, AC004080, AC010990. Human HoxB, $H o x \mathrm{C}$, and HoxD clusters were taken from genomic DNA from the UCSC genome browser http://genome.ucsc.edu/ (NCBI35/hg17, release May 2004). The HoxB cluster is taken from chromosome 17, nucleotides: 43,961,813-44,161,040. The HoxC cluster is taken from chromosome 12, nucleotides: $52,618,296-52,735,253$. The HoxD cluster is taken from chromosome 2 , nucleotides: 176,772,385-176,881,142.

Xenopus tropicalis (frog) Hox clusters were taken from Ensembl web browser http://nov2005.archive.ensembl.org/Xenopus_tropicalis/index.html (JGI 3, release Jan 2005 v35.1b). The HoxA cluster is taken from scaffold 29 nucleotides: 1,777,7892,133,531. The HoxB cluster is taken from scaffold 329 nucleotides: 415,000-1,016,000. The HoxC cluster is taken from scaffold 280 nucleotides: 199,492-581,365. The HoxD cluster is taken from scaffold 353 nucleotides: 474,676-800,000.

Latimeria menadoensis (coelacanth) HoxA cluster from EVX1 to HoxA9 was taken from BAC clone VMRC4-19C10 with genbank accession number 147788. The rest of the $H o x \mathrm{~A}$ cluster and the entire $H o x \mathrm{~B}, \mathrm{C}$, and D clusters were isolated from BAC clones and sequenced.

Heterodontus francisci (horn shark) HoxA cluster was constructed from two clones with accession numbers AF479755 and AF224262. The HoxD cluster was a clone with accession number AF224263.

Polypterus senegalus (bichir) HoxA cluster was made from two clones - 22 F 22 and $-164 C 2$ with accession numbers AC 126321 and AC 132195 , respectively. The partial Hox B cluster is constructed from clone L28995. Hox B 13 is on segment L28995.23. Hox B 10 is on segment L 28995.7 . Hox B 8 and Hox B 7 are on segment L28995.17. Hox B 5 and Hox B 4 are on segment L28995.1. The partial Hox D cluster is constructed from clone CLN84 which includes HoxD3, HoxD2, and HoxD1.

Danio rerio (zebrafish) Hox clusters were made from clones. HoxAa cluster was constructed completely from clone 241I17 accession number AC107364, modifications included trimming the first 26,896 and the last 4,176 nucleotides and inserting a C at position 76,071 . The HoxAb cluster was made from clone 100019 accession number AC107365 with an alteration of nucleotide 79,324 from T to C to avoid a premature stop codon. The HoxBa cluster was constructed with two clones CH211-72A16 and BUSM1254 O 17 with accession numbers BX927395 and AL645782, respectively. Clone CH21172A16 was spliced to BUSM1-254O17 immediately after the HoxB4a gene. The HoxBb cluster was simply clone BUSM1-227H09 accession number AL645798 used in its entirety with no alterations. The HoxCa cluster was made from clones DKEY 148F24 and clone DKEY 81P22 with accession numbers BX465864 and BX005254, respectively. The $H o x \mathrm{Cb}$ cluster was taken from a scaffold from the Ensembl genome browser http://nov2005.archive.ensembl.org/Danio_rerio/index.html (WTSI Zv5, release July 2005 35.5b). The HoxDa cluster was clone RP71-78H1 accession number BX322661 used in its entirety.

Oreochromis niloticus (nile tilapia) HoxA cluster is the entire clone with accession number AF533976.

Morone saxatilis (striped bass) HoxA cluster is the entire clone with accession number AF089743.

Oryzias latipes (medaka) Hox clusters were taken from genbank in their entirety. Нox $\mathrm{Aa}, \mathrm{Ab}, \mathrm{Ba}, \mathrm{Bb}, \mathrm{Ca}, \mathrm{Da}$, and Db are under accession numbers $\mathrm{AB} 232918-\mathrm{AB} 232924$, respectively.

Tetraodon nigroviridis (spotted-green pufferfish) Hox clusters were all taken from Tetraodon Genome Browser at http://genome.ucsc.edu (Genoscope 7/tetNig1, release Feb 2004). The HoxAa cluster is taken from chromosome 21, nucleotides: 2,878,001$3,153,406$. The HoxAb cluster is taken from chromosome 8 , nucleotides: 6,506,4716,727,504. The HoxBa cluster is taken from chromosome Unknown, nucleotides: 37,928,410-38,293,032. The HoxBb cluster is taken from chromosome 2, nucleotides: 1,321,876-1,537,033. The HoxC cluster is taken from chromosome 9, nucleotides: 4,083,941-4,353,227. The HoxDa cluster is taken from chromosome 2, nucleotides: 10,975,763-11,218,409. An alteration was made at nucleotide position 11,134,740; a T was deleted in order to shift back to correct frame. The HoxDb cluster is taken from chromosome 17, nucleotides: 9,471,355-9,694,740.

Takifugu rubripes (Japanese pufferfish) Hox clusters were acquired from the http://nov2005.archive.ensembl.org/Fugu_rubripes/index.html (FUGU 2.0, release May $2004 \mathrm{v} 35.2 \mathrm{~g})$. The HoxAa cluster is constructed from the entire scaffold 47. The HoxAb cluster is constructed from scaffold 330.

### 3.2.3 Create Conserved Non-coding Sequence Database

To obtain PFCs, pair-wise alignments were made between orthologous intergenic regions of human Hox clusters and all other species Hox clusters, bichir Hox clusters and
all ray-finned fish Hox clusters, and among all teleost Hox clusters using MacVector version 8.0 (Accelrys ${ }^{\circledR}$ ) which uses the clustalW algorithm. For PFC analysis the pairwise alignments between acanthomorph 'a' paralog Hox clusters were excluded due to their overall high identity. PFCs in this study were defined by the canonical definition of 2 or more sequences with $100 \%$ identity over at least 6 base pairs each of which are within 100 base pairs of each other. This analysis consisted of over 900 pair-wise alignments of homologous intergenic regions with an average alignment size of 10 kilobases. Due to the large number of PFCs obtained by this method I further restricted the criteria to include the alignment of each pair-wise PFC to be at least $60 \%$ identical and/or contain at least 5 PFs. All Hox clusters used in this study were scanned for repetitive and mobile DNA elements using the RepeatMasker database at http://www.repeatmasker.org/. PFCs that corresponded to masked elements were excluded.

In total I identified 563 unique PFCs that fit my criteria. After this I carefully determined which pair-wise PFCs overlapped across other species and numbered according to the relative position in the cluster with 1 starting at the posterior end of the cluster and ascending towards the anterior end of the cluster. This was accomplished by starting with the 'seed' species of human, bichir and zebrafish and determining overlapping PFCs by their overlapping nucleotide positions. After overlapping PFCs were determined, any PFC that existed in more than 2 species was subjected to a multiple alignment that was then trimmed to maintain the criteria of a PFC (Table S1).

PFCs were then broken down into sections based on differential retention of that section in the species that contain that PFC. A section may be as small as an individual

PF or as large as all but one PF. The PFCs were named based on their location in the cluster and for PFCs conserved among more than 2 species, their section content. The name consists of a 3 letter abbreviation for the species that it is found in, followed by 1 or 2 capital letters denoting the Hox cluster it resides in, followed by a 2 letter subscript denoting the intergenic region it resides in (see below), followed by the number of the PFC which are roughly ordered by their position along the cluster starting at the $5^{\prime}$ 'end and sometimes followed by a 1-6 letter subscript describing the section content of that PFC (e.g. a PFC that is found between HoxA9 and HoxA8 in human is named HsaA $\mathrm{AH}_{\mathrm{GH}} 74_{\text {abe }}$ which signifies that it is found in human between genes A 9 and A 8 , it is the $74^{\text {th }}$ PFC on the HoxA cluster and it shares sections a, b, and e with other species; Figure 8). For nomenclature purposes Hox genes were coded A-O starting with EVX and ending with group 1 genes. We propose that if new PFCs are found within a Hox cluster used in this analysis that they be named and numbered based on the following formula:

$$
X=\frac{\operatorname{distAP}}{\operatorname{distAB}}+A
$$

$\mathrm{X}=$ number of new PFC
$\mathrm{P}=$ new PFC
$\mathrm{A}=\mathrm{PFC} 5$ ' to P
$B=P F C 3$ ' to P
(e.g. if a new PFC is found 430bp downstream of $\mathrm{HsaA}_{\mathrm{GI}} 103$ and $\mathrm{HsaA}_{\mathrm{GI}} 104$ is 670bp downstream, then the new PFC should be named $\mathrm{HsaA}_{\mathrm{GI}} 103.64$ ). I then compiled this information into two types of charts. One containing the name, location and sequence of PFCs in each individual Hox cluster and the other containing all PFCs in one Hox cluster paralog.

### 3.2.4 Analyze Conserved Non-coding Sequence Database

After the PFC database was created we searched for retention and loss patterns that may be pertinent to the evolution of gnathostomes with special emphasis on the rayfinned fishes and teleosts.

The first pattern looked at was 'deeply' conserved PFCs. 'Deeply' conserved PFCs are defined as found in at least 2 of the following groups, 1) horn shark, 2 ) $\geq 2$ lobefinned fish, 3) bichir, 4) $\geq 2$ teleost fish. This criterion limits 'deeply' conserved PFCs to those that have maintained identity over at least 733 million years of additive evolutionary time. Of the 563 unique PFCs, 78 (13.9\%) fit this criterion (Figure 9). Given a random distribution and an average PFC divergence time of at least 270 million years (low end estimate) one would expect $37 \%$ of the PFCs to fit the criteria of at least 733 million years so this is a significantly small amount ( p -value $=0.001$ ). All PFCs were blasted against the expressed sequence tag (EST) database at http://www.ncbi.nlm.nih.gov/BLAST/. If human or zebrafish contained the PFC then their sequence was preferentially used to perform the blast search. All PFCs were also blasted against the nr database to identify untranslated regions and microRNAs. Additionally, PFCs considered 'deeply conserved' were blasted against the non-coding RNA database at http://research.imb.uq.edu.au/rnadb/default.aspx (Pang et al. 2005; Table S2).

Literature searches were done to identify all known Hox CREs and micro-RNAs. All known Hox cis-regulatory elements and micro-RNAs were detected as deeply conserved PFCs. This speaks to the robustness of this method at detecting conserved functional non-coding sequences. To help determine those PFCs that would be good
candidates for cis-regulatory elements efforts were made to identify PFCs that existed within sequences of known RNA transcripts. Of the 78 'deeply' conserved PFCs, 8 corresponded to sequences of known function: C8 early enhancer (Shashikant et al. 1995), A2 enhancer (Nonchev et al. 1996), D4 retinoic acid response element (RARE; Morrison et al. 1996), A4 RARE (Doersken et al. 1996), A5 MES enhancer (Larochelle et al. 1999), mir-196b (Yekta et al. 2004), mir-196-2 (Berezikov et al. 2005) and mir-10b (Berezikov et al. 2005). Of the remaining 70 'deeply' conserved PFCs, 22 were found in untranslated regions (UTR) of the RNA transcripts of Hox genes, 21 were found in the expressed sequence tag (EST) database and 27 have an unknown function. Of those 27, 6 lie within 500 bp up or downstream of the coding sequence of a Hox gene, which implies they could possibly be part of an unsequenced UTR or basal promoter element. That leaves 21 'deeply' conserved PFCs that could possibly act as cis-regulatory elements. It is also possible that those PFCs that lie within RNA transcripts and UTRs could also serve a cis-regulatory role.

Deeply conserved PFCs seem to have a biased towards the 3' end of the cluster. Between the paralogous Hox clusters the only difference that is apparent is the seemingly few deeply conserved PFCs on the HoxC cluster which is mainly due to the lack of PFCs with no known function. This may be due to sampling bias as the HoxC cluster is the only cluster that contains only 2 of the 4 groups considered (see figure 9 for explanation of groups). The finding of deeply conserved PFCs are not the only pattern worth exploring however, many unique patterns can be found when the phylogeny of the species used in this study are considered.

Several patterns of retention/loss of PFCs throughout gnathostome phylogeny can be seen in this database. First we will address the previous findings of Chiu et al. (2004), where it was determined that bichir showed a pattern of 'mosaicism' in its PFC content.

Previous studies found a trend of mosaicism in the retention of PFCs on the HoxA cluster of bichir when comparing it to the HoxA clusters of non-ray finned fish and teleosts, the more derived ray-finned fish clade. This mosaicism showed alternatives to the apparent pronounced loss of PFCs in teleost fishes, as seen by Chiu et al. 2002, when comparing them to the HoxA clusters of human and horn shark whose Hox clusters shared a remarkable number of PFCs. In the current study the HoxA cluster of bichir shared 17 PFCs exclusively with non-ray finned fishes (lobe-finned fishes and cartilaginous fishes) and 48 PFCs exclusively with teleost fishes which is $26 \%$ and $74 \%$ respectively.

This appears to be in contrast with the numbers shown in Chiu et al. 2004 because bichir shares much more with the teleosts but still supports the claim of bichir showing a mosaic pattern between non-ray finned fishes and teleosts. When doing this comparison however it is important to take into consideration the number of Hox clusters compared in each category. When scaled by the number of pair-wise comparisons made the numbers are a little closer with $47 \%$ of the PFCs found exclusively between bichir and non-ray finned fishes and $53 \%$ found between bichir and teleosts.

The pattern is different for the HoxB cluster where bichir shared $21 \%$ of its PFCs with the non-ray finned fishes and $79 \%$ of its PFCs with the teleosts even after scaling for number of pair-wise comparisons (Table 4). In order to see if this pattern may be by chance and also to assess the amount of conservation in the derived ray-finned fishes we
also calculated the number of PFCs shared exclusively between human and other non-ray finned fishes and human and teleosts. After scaling, the HoxA cluster of human shared $74 \%$ of its exclusive PFCs with other non-ray finned fishes and $26 \%$ with teleosts. These numbers were comparable on the HoxB cluster with $82 \%$ and $18 \%$ respectively, again showing the conservative nature of non-ray finned fishes Hox clusters.

Another category needed to determine the true mosaicism of bichir's Hox clusters is the number of PFCs that are shared only among the teleosts species. This becomes a problem however because the Acanthomorpha, which consists of all the teleosts in this study excluding zebrafish, are too closely related. This makes it difficult to assign PFCs because of the high identity between their non-coding regions. For this reason the category of "exclusive to teleosts" must be reduced to "shared exclusively between zebrafish and any other teleosts".

The HoxAa cluster of zebrafish shared $49 \%$ of its PFCs with non-teleosts (lobe finned fishes, cartilaginous fishes and basal ray-finned fishes) and $51 \%$ with teleosts with or without scaling. The HoxAb cluster was $71 \%$ non-teleosts and $29 \%$ teleosts; HoxBa $29 \%$ non-teleosts and $71 \%$ teleosts; $H o x \mathrm{Bb} 56 \%$ non-teleosts and $44 \%$ teleosts all with scaling. For the HoxAa cluster andBb cluster zebrafish appears completely mosaic. For the $H o x A b$ cluster zebrafish appears to be more non-teleost like and for the HoxBa cluster zebrafish appears to be more teleost like. If the ' $b$ ' paralog clusters are ignored due to their low number of PFCs (and reduced gene number) then the zebrafish takes on a cluster specific pattern that mirrors that of bichir. The HoxA cluster of bichir and its ortholog the HoxAa cluster of zebrafish both share around $50 \%$ with non-teleosts and $50 \%$ with teleosts. In contrast to the HoxA cluster but in agreement with one another, the

Hox B cluster of bichir and the Hox Ba cluster of zebrafish both share around $25 \%$ with non-teleosts and $75 \%$ with teleosts (Figure 10).

These patterns and others that will be explained in the following paragraphs show evidence that not only do bichir Hox clusters show 'mosaic' patterns but also these patterns are different for different clusters. When one combines this data of turnover in the ray-finned fishes (starting in bichir and continuing into teleosts) with the data that show the lack of retention of ray-finned fish specific PFCs it highlights the dynamic genomic environment that exists in the teleosts that started to take root in the basal ray finned fishes. This is in stark contrast to the extremely conserved nature of the Hox cluster regulatory environment seen in and among the sister lobe-finned fishes and more basal cartilaginous fishes.

Combining the information of deeply conserved PFCs with the idea of looking for patterns of gain/retention/loss in the Hox clusters we constructed a phylogenetic tree with apparent acquisition and loss numbers for deeply conserved PFCs at each node (Figure 11). The criteria of a deeply conserved PFC requires that the PFC has been conserved for $\sim 1$ billion years of additive evolutionary time making this type of analysis very important in identifying large (time) scale changes in non-coding sequence conservation within the Hox clusters of the gnathostomes. The identity of whether the PFC was gained or lost in each specific lineage was determined on a parsimony basis. Currently only the HoxA cluster can be analyzed using this method because it is the only cluster that we have complete sequences for in horn shark and bichir, two pivotal species in the analysis (Figure 11).

For the HoxA cluster it is evident that at least 30 PFCs were acquired sometime before the divergence of the 3 major gnathostome lineages and differential loss of these PFCs can be seen with the majority being absent in the teleosts' HoxAb cluster. 4 PFCs were gained sometime after the divergence of cartilaginous fish but prior to the divergence of the lobe-finned and ray-finned lineages. Again the majority of these are absent in the teleosts' HoxAb cluster. The lobe-finned fish lineage appears to have acquired 2 PFCs at the base which have been maintained throughout lobe-finned phylogeny. The lobe-finned fishes have also maintained all but one of the gnathostome acquired PFCs. The ray-finned fish lineage appears to have gained 7 new PFCs after they diverged from the lobe-finned fishes. They also lost two of the gnathostome PFCs. Bichir does not appear to have lost any of the gnathostome or bony-fish PFCs. The teleosts appears to have lost 7 more of the gnathostome PFCs and 2 of the bony-fish PFCs sometime prior to the genome duplication and did not maintain any PFCs that were gained after the divergence of bichir. The HoxAa cluster is more conservative than the Hox Ab cluster in that it only lost 1 more of both the gnathostome PFCs and ray-fin PFCs after the genome duplication. The HoxAb cluster appears to be very degenerate in its PFC conservation in that it lost 14 additional gnathostome PFCs, 1 additional bony-fish PFC, and 5 additional ray-fin fish PFCs after the genome duplication. Also the HoxAa and HoxAb clusters appear to have gained 1 PFC each after the genome duplication. It is equally parsimonious to assume that each of these PFCs was gained after bichir diverged but sometime prior to the genome duplication and then subsequently lost in the other paralogous cluster.

These results highlight the turnover of highly and anciently conserved PFCs (putative CREs) that began at the base of the ray-finned fish clade and continued through the derived ray-finned fish clade and the lack of turnover in the lobe-finned and cartilaginous clades.

### 3.3 Mutation Rate Estimation

## Materials and Methods

Intergenic distances analysis was done on select intergenic sequences. I chose intergenic sequences from the $5^{\prime}$ and $3^{\prime}$ end of each of the 4 Hox clusters based on the availability in phylogenetically important taxa. The intergenic sequences chosen for analysis were sequences between HoxA13 and HoxA11, HoxA5 and HoxA4, HoxB9 and HoxB8, HoxB3 and HoxB2, HoxC12 and HoxC11, HoxC8 and HoxC6, HoxD12 and HoxD11, and HoxD4 and HoxD3. Comparisons were made between teleost fishes; Japanese pufferfish (Tru) and spotted green pufferfish (Tni), medaka (Ola), and zebrafish (Dre). Comparisons were then made between mammal species with approximately similar divergence dates; human (Hsa) and baboon (Papio hamadryas, Pha), dog (Cfa), and opossum (Monodelphis domesticus, Mdo). Intergenic sequences were aligned using clustalW algorithm in MacVector version 9.0 (MacVector, Inc.). Jukes-Cantor D-values were calculated from these alignments using Mega version 3.1.

Approximate divergence times between two species that are necessary when performing comparative studies for conservation of non-coding DNA sequences can differ depending on generation times and mutation rates of the taxa selected. For mammals a general time of at least 250 million years divergence was determined by Tagle et al. (1988) for performing phylogenetic footprinting analysis. For other taxa divergence times have not been well worked out. In this study most of the species comparisons fit into the criteria of at least 250 million years divergence except for the comparisons among teleosts (zebrafish, acanthomorphs). For this study it appeared that the intergenic regions of the Hox clusters of the acanthomorph species (striped bass, nile
tilapia, pufferfishes; diverged $\sim 80-100$ mya) were too similar to do a phylogenetic footprint comparison but when aligned with zebrafish which diverged $\sim 135$ million years ago (mya) from the acanthomorphs the intergenic regions appeared to be dissimilar enough to do phylogenetic footprint comparisons. For this reason we decided to compare intergenic region distances between teleosts with a mammalian proxy to see if indeed their intergenic regions were diverging quicker.

Figure 12 shows the Jukes-Cantor D-values for teleosts fish versus their mammalian proxies. Immediately apparent is the lesser values for the mammalian proxies which implies that either the teleosts have a much higher mutation rate, much shorter generation time, or the proxies are incorrect due to inaccurate divergence time estimates. The D-values of zebrafish versus Japanese pufferfish allowed us to determine that these species were divergent enough to perform a phylogenetic footprinting analysis. Therefore pair-wise alignments containing zebrafish versus the acanthomorphs were included despite the relatively recent divergence date.

Another pattern that is not as easily noticeable is the difference between the 5 , and 3 ' ends of the clusters. The odd numbers which correspond to the 5' ends of the clusters all have greater D-values than the even numbers which correspond to the $3^{\prime}$ ends of the cluster. This greater conservation at the 3 ' end is also noticeable in Figure 3 where the deeply conserved PFCs are biased towards the 3' end. This data supports the posterior flexibility hypothesis put forward by Ferrier et al. (2000) which states that the posterior Hox genes (9-14) are evolving faster than the anterior (1-8) and implies this is occurring, in general, at the posterior end of the Hox gene cluster.

### 3.4 Conclusion

Evolution at the most basic level is change in allele frequency that becomes fixed over time. Empirical and theoretical evidence suggest that selection at the population level is most likely to occur in cis-regulatory elements. Because of this it is important to understand the code and evolution of these non-coding elements. Without a standardized code for cis-regulatory elements and since they do not code for an identifiable alternative molecule, their identification is not as straightforward as it is for coding DNA.

In an effort to uncover these molecular gems among the other DNA, unique methods have been developed. One such method is detection of evolutionary conservation of non-coding DNA. This method has proved to be advantageous and accurate although not without weaknesses or hurdles. One such hurdle is the fact that sequence conservation may not be necessary for functional conservation (Fisher et al. 2006). Unfornutately, this hurdle cannot currently be overcome. Another such hurdle is finding homologous non-coding sequences to compare since by definition the DNA surrounding the CNSs must not be conserved. A unique molecular phenomenon that can help overcome this issue is the clustering of genes that remains intact over millions of years in disparate species resulting in stretches of definitive homologous non-coding DNA. One such system is the Hox gene cluster.

Despite the convenience, few authors attempt to find CNSs within the Hox gene clusters and those that have, concentrate on only a few species. In this study, we have done a complete scan of gnathostome Hox gene clusters' intergenic regions to identify as many putative CREs as possible while gaining insight into the evolution of these
sequences in jawed-vertebrates with specific focus on the ray-finned fishes and the highly speciose, diverse and derived group, teleosts.

First, it is apparent that CNSs in the gnathostome Hox gene clusters adhere to the posterior flexibility hypothesis of Hox genes put forth by Ferrier et al. (2000). Second, we have extended the work done by Chiu et al. (2004) showing the mosaicism in basal ray-finned fishes Hox gene clusters. The complete scan of gnathostome Hox clusters has revealed additional data supporting this mosaicism but has also shown that the turnover that causes this mosaicism has been continuous throughout the ray-finned fishes from the basal bichir to the highly derived teleosts family, Percomorpha. This is shown by the fact there are no CNSs that are conserved exclusively in teleosts. Zebrafish, an intermediate teleost, appears to be as mosaic as bichir when comparing its conservation to species that diverged before to that of species that diverged after. The turnover of CNSs contributing to this mosaicism is cluster specific indicating that the Hox gene cluster system does not necessarily evolve under the same pressures and circumstances across all clusters. Third, in an effort to formalize the identification of CNSs within the Hox gene cluster system we have proposed a flexible naming system as Scott (1993) did for the Hox genes.

## Chapter 4- Defining a Cause of Uniqueness

### 4.1 Introduction

### 4.1.1 Hindbrain Development

What is happening at the molecular and population level that may explain this turnover in conserved non-coding sequences in ray-finned fishes? To help answer this question we have turned to a well-characterized enhancer controlling the expression of HoxA2 in the developing hindbrain. We will start with a brief introduction of hindbrain development and the known expression of HoxA2 in vertebrates. We will then perform both macro- and micro-evolutionary studies of the enhancer to help determine possible mechanisms of its seemingly dynamic expression patterns in teleosts.

The vertebrate central nervous system (CNS) develops from neuroectoderm that thickens into the neural plate that rolls into the neural tube. This process occurs in an anterior to posterior pattern. The CNS is ultimately divided into the forebrain, midbrain, hindbrain and spinal cord. During the development of the hindbrain the neural tube is divided into distinct segments called rhombomeres much like the mesoderm is divided into distinct segments termed somites. Hox genes are expressed in the developing CNS starting with an anterior border at rhombomere 2 (r2) in the hindbrain and ending at the posterior end of the spinal cord. More specifically group 1-4 genes are restricted to anterior expression limits in the hindbrain whereas group 5-13 genes all have an anterior limit in the spinal cord (Gaunt et al. 1989; Hunt et al. 1991; Keynes and Krumlauf 1994). The peripheral nervous system (PNS) develops from a group of cells that is derived from an area between the thickened neural plate and the overlying ectoderm called the neural crest. Neural crest cells migrate from a dorsal to ventral position and convert from
neuroectoderm into mesenchymal cells which give rise to numerous structures. Pharyngeal arches (or branchial arches in fish) develop from these migrating neural crest cells. The first pharyngeal arch (PA1) arises from r 2 , the second pharyngeal arch (PA2) arises from r 4 and the third pharyngeal arch arises from r6. The first pharyngeal arch will become the lower oral jaw and the second pharyngeal arch will become the hyoid in tetrapods and structures supporting the lower oral jaw in fish. Pharyngeal arches 3-6 or 7 become parts of the pharynx and trachea in tetrapods and gills and pharyngeal jaws in fish.

### 4.1.2 Hox A2 Expression

HoxA2 is expressed in the developing hindbrain with an anterior limit of expression at the $\mathrm{r} 1 / \mathrm{r} 2$ boundary and continues to be expressed through r 7 in mouse and chick embryos (Hunt et al. 1991; Krumlauf 1993; Prince et al 1994; Figure 13). Mouse HoxA2 has stronger expression in r3, 5 whereas chick has strong expression r3-7. Immediately following the FSGD there were two HoxA2 paralogs dubbed HoxA2a and HoxA2b. Since the FSGD, both paralogs have been independently pseudogenized in fish with a MRCA of 135 mya (zebrafish HoxA2a, Prince et al. 1998; medaka HoxA2b, Davis et al. 2008) as well as maintained as paralogs with differing expression patterns in fish with a MRCA of $\sim 100$ mya (Japanese pufferfish, Amores et al. 2004; Tumpel et al. 2006; striped bass, Scemama et al. 2006; Nile tilapia, Le Pabic et al. 2007). Zebrafish HoxA2b gene maintains a near identical ancestral expression pattern in r2-5 with stronger expression in r2, 3 (Prince et al. 1998). The same is true for the HoxA2b gene of Japanese pufferfish (Amores et al. 2004, Tumpel et al. 2006). The HoxA2a gene of Japanese pufferfish however has lost almost all hindbrain expression and appears in a
small stripe of cells in r1, 2. Striped bass and Nile tilapia HoxA2a genes are expressed in r2-7 with stronger expression in $\mathrm{r} 2,3$ similar to the HoxA2b genes of zebrafish and pufferfish (Scemama et al. 2006, LePabic et al. 2007). The HoxA2b genes of striped bass and Nile tilapia are more weakly expressed. Striped bass HoxA2b gene is expressed in r2-5 and Nile tilapia HoxA2b is expressed in r2-5, 7 with stronger expression in r3, 5 (Scemama et al. 2006; LePabic et al. 2007; Figure 14).

HoxA2 is also expressed in the peripheral nervous system (PNS), specifically neural crest cells derived from r4 that pattern PA2 (Hunt et al. 1991; Krumlauf 1993; Prince et al 1994). This expression is maintained among tetrapods HoxA2 genes but again has various patterns in the teleosts' HoxA2 paralogs. In zebrafish, which has a pseudogenized HoxA2a, only the HoxA2b paralog is expressed and maintained in neural crest cells (Prince et al. 1998). The same is true for pufferfish HoxA2b expression (Amores et al. 2004; Tumpel et al. 2006). In striped bass and Nile tilapia, both paralogs are expressed weakly at the onset but only HoxA2a is maintained in detectable levels (Scemama et al. 2006, LePabic et al. 2007). Medaka, which has a pseudogenized HoxA2b, follows the same pattern as striped bass and Nile tilapia (Davis et al. 2008).

Controlling the expression of HoxA2 genes are 3 distinct enhancers (Figure 15). Expression in r 2 is controlled by an enhancer in exon 2 of the HoxA2 gene, expression in r 4 is controlled by an enhancer within the intron of the HoxA2 gene, and expression in r3, 5 and in r 4 derived neural crest cells is controlled by an enhancer $\sim 1-3 \mathrm{~kb}$ upstream of the HoxA2 gene transcription initiation site (Nonchev et al. 1996; Maconochie et al. 2001; Tumpel et al. 2006). Within each of these enhancers are distinct regulatory elements that drive partial expression.

The r3/5 enhancer is the only known HoxA2 enhancer that lies outside the transcriptional start and stop sites. The r3/5 enhancer is divided into 5 elements that control rhombomere expression that are known as 'response elements' (RE1-5, Nonchev et al. 1996; Maconochie et al. 2001; Tumpel et al. 2006) and 4 elements that control neural crest and pharyngeal arch expression that are known as 'neural crest elements' (NC1-4, Maconochie et al. 1999). Some upstream transcription factors have been identified that bind to these cis-regulatory elements and are necessary for proper HoxA2 expression and rhombomere and pharyngeal arch development. There appears to be 1 specific transcription factor for each of the three compartments where HoxA2 expression is driven by the $\mathrm{r} 3 / 5$ enhancer. Krox-20 is normally expressed in tissue that will become rhombomeres 3 and 5 (Wilkinson et al. 1989; Nieto et al. 1991; Bradley et al. 1993; Oxtoby and Jowett 1993). Krox-20 (a.k.a. Kruppel, egr2) has two binding sites in the r3/5 enhancer sequence and directly regulates expression of HoxA2 in r3 and partially in r5 (Nonchev et al. 1996). Mafb (a.ka. Kreisler, valentino) is normally expressed in and is necessary for $\mathrm{r} 5 / 6$ development (Prince et al. 1998b; Sadl et al. 2003). Mafb has multiple binding sites within the r3/5 enhancer and though it has not been shown that it directly regulates HoxA2, HoxA2 expression is absent in r 5 in Mafb ${ }^{(--)}$zebrafish (Prince et al. 1998b). AP-2 is expressed in the neural crest cells and their derivatives (Mitchell et al. 1991). AP-2 has multiple binding sites in the r3/5 enhancer and, though it has not been shown to directly regulate HoxA2 expression most likely due to redundant AP-2 family members, the $\mathrm{r} 3 / 5$ enhancer has been shown to drive expression of a reporter gene in the same pattern as AP-2 expression (Maconochie et al. 1999). The combination of these 3 main transcription factors and several co-factors that have not yet been identified are
responsible for the total expression of HoxA2 in r3 and 5 and neural crest cells derived from r 4 in the developing hindbrain of all studied vertebrates.

The r3/5 enhancer has been studied in much detail via deletion and swap assays. Deletion assays in mouse done by Nonchev et al. (1996), Maconochie et al. (1999) and Maconochie et al. (2001) identified RE1-4 and NC1-4 as well as two essential Krox-20 binding sites and an element known as Box A. Both Krox-20 sites, Box A and RE1-3 are necessary for proper expression in r3 and 5 although r5 expression is only lost completely in Krox-20 site deletions (Maconochie et al. 2001). RE4 is shown to stimulate r5 expression in the presence of Krox-20 sites. Both RE1 and RE3 contain a TCT motif (TCTNAC) that appears to be the essential binding site for their function (Maconochie et al. 2001). A similar experiment was done for expression in the neural crest cells. NC1, 3 and 4 were shown to be essential for neural crest cell expression but did not affect r3, 5 expression (Maconochie et al. 1999). NC2 was shown to be essential for neural crest cell expression and although it did not affect r 3 expression it did lower expression in r 5 (Maconochie et al. 1999). It is important to note that NC2 and RE4 overlap as well as NC3 and RE3. NC3 and RE3 do not appear to overlap in essential positions though because absence of NC3 does not abolish r3, 5 expression like absence of RE3 (Maconochie et al. 1999; Maconochie et al. 2001). Most likely it is the TCT motif of RE3 that is responsible for driving expression in $\mathrm{r} 3,5$. NC2 and RE4 both give a similar phenotype of reducing or ablating expression in r5.

Swap assays were done in the Japanese pufferfish (Tumpel et al. 2006). The Japanese pufferfish has both a HoxA2a and HoxA2b paralog although the HoxA2a paralog has lost almost all expression (Amores et al. 2004). Tumpel et al. (2006)
assumed that the loss of expression of HoxA2a was due to inactive enhancer elements.
To take advantage of this they swapped out RE1-4 individually from the functional $H o x \mathrm{~A} 2 \mathrm{~b}$ r3/5 enhancer into the non-functional HoxA2a r3/5 enhancer to determine how much each individual response element contributed to the overall r3, 5 expression levels. From this analysis they determined the fraction of embryos each response element was able to drive expression in and decided that this was the efficiency of that particular element. The results were as follows: Box A 0\%, RE2 6\%, RE3 67\%, RE4 32\% and a newly discovered RE5 33\%. Although this percentage was calculated based on the fraction of embryos, the intensity of the staining was directly correlated. Combining these analyses it shows that RE3 is a major factor in r3, 5 expression whereas RE4 and RE5 are minor factors.

### 4.2 Expression Analysis of Hox A2 Paralogs

## Materials and Methods

We used in situ hybridization using mRNA probes on whole mounted embryo to detect expression of HoxA2a and HoxA2b in a tissue and stage specific manner. HoxA2a, HoxA2b and EGR2 were isolated from F. heteroclitus gDNA (gift from D. Duvernell) using PCR protocol as described in section 2.2.1 with the following modifications: HoxA2a was isolated with HoxA2a exon 2 forward (5'-

TGACCGAGAGGCAGGTCAAGG-3') and HoxA2a exon 2 reverse (5'-AGGGCAGAGGGGCTGTCACC-3') primers at an annealing temperature of 56 C; HoxA2b was isolated with HoxA2b forward (5'-CCTGACATCTCTCGCTAACC-3') and HoxA2b reverse (5'-AGAGGTCAGACGCTGCTGC-3') primers at an annealing temperature of 59 C; EGR2 was isolated with EGR2 forward (5'-CCAGACCTTYACCTAYATGGG-3') and EGR2 reverse (5'-TGTGTCTCTTYCTCTCRTCGC-3') primers at an annealing temperature of 59 C to yield a 400 bp , 250bp and 955 bp fragment, respectively. DNA fragments were cloned into the pGEM T-vector (Promega). RNA probes were synthesized using Roche T7 RNA polymerase (10881767001) that primes from the pGEM T-vector T7 site. HoxA2a andA2b RNA were synthesized using Roche DIG RNA labeling mix (1127703910). EGR2 was synthesized using Roche Fluoroscein RNA labeling mix (11685619910). RNA was purified using standard Phenol/Chloroform extraction. In-situ hybridizations were performed as described (Thisse and Thisse 2008) with the following modifications. Embryos were de-chorionated and de-yolked after rehydration and before digestion. Embryos were then digested in proteinase K for 50 seconds per somite. Antibodies were
pre-absorbed in blocking buffer for 2 hours at 4 C while rocking. Following overnight incubation with antibody, embryos were washed 6 times for 30 min each with gentle agitation. Embryos were stained in ceramic spot plates in the dark.

For two-color staining, both EGR2 and the HoxA2(a or b) RNA probe were hybridized to the embryo simultaneously. The $1^{\text {st }}$ stain was conducted for EGR2 via Roche anti-fluorscein-AP Fab fragments with Sigma Fast ${ }^{T M}$ FastRed (Sigma). The embryo was then washed 2 times for 30 minutes each in 1X PBT followed by a 30 minute wash in AP inactivation solution ( 0.1 M glycine- $\mathrm{HCl} \mathrm{pH} 2.2,0.1 \%$ Tween 20), followed by 4 times for 30 minutes in 1XPBT. The $2^{\text {nd }}$ stain was conducted for HoxA2(a or b) via Roche anti-DIG-AP Fab fragments with NBT/BCIP stain providing a contrasting blue/purple color. Stained embryos were imaged using a Leica DFC290 digital camera attached to a Leica MZ12.5 stereo dissecting microscope.

In-situ hybridization analysis was performed with mRNA of HoxA2a and HoxA2b genes of mummichog (Fundulus heteroclitus) on $F$. heteroclitus embryos from 4 distinct developmental stages to determine the pattern of expression the Fundulus $s p$. group has compared to the patterns seen in other teleosts lineages. Both the HoxA2a and HoxA2b gene have a similar onset of expression in $\mathrm{r} 2,3$ seen in figures $16 \mathrm{~m}, \mathrm{n}$ respectively. At the 8 -somite stage $H o x A 2 a$ and $H o x A 2 b$ show weak expression in r2-4 and PA2 (Figures 16a-d). The strongest expression is seen at the 14-somite stage (as is in other teleosts; Prince et al. 1994; Scemama et al. 2006; Le Pabic et al. 2007; Davis et al. 2008) where HoxA2a is expressed in r2-7 with stronger expression in the more anterior rhombomeres and HoxA2b in r2-5 with weak expression in r5 (Figures 16e-h). In difference to other teleosts both HoxA2a and HoxA2b are strongly expressed in PA2 at
the 14 -somite stage (Figures $16 \mathrm{~g}, \mathrm{~h}$ ). Expression was also detected at the 21 -somite stage to determine maintenance of expression. At this stage $H o x A 2 a$ and $H o x A 2 b$ are again similar with expression in r2-5 and PA2 although HoxA2b appears to have slightly stronger expression in PA2 (Figures 16i-1).

Compared to other teleosts $F$. heteroclitus maintains the ancestral expression of the HoxA2a gene. This is deduced from known expression data where striped bass, Nile tilapia and medaka all have expression in r2-7 with stronger expression more anteriorly and also strong expression in PA2 (Scemama et al. 2006; Le Pabic et al. 2007; Davis et al. 2008). This assumes that the psuedogenization of HoxA2a in zebrafish and the near complete loss of expression in Japanese pufferfish were independent events, which is the most parsimonious conclusion (Prince et al. 1998a; Amores et al. 2004). The HoxA2b paralog however shows continued variation that is seen in other teleosts. In $F$. heteroclitus the HoxA2b gene is expressed strongly in r2-4 but weaker in r5 (Figure 16f, h). For the other known teleosts expressions, striped bass has weak expression in r2-5, Nile tilapia has weak expression in $\mathrm{r} 2,4,7$ and strong expression in r 3 , 5 , medaka has a pseudogene, Japanese pufferfish has strong expression in r2-5 and zebrafish has weak expression in r4, 5 and strong expression in r2, 3 (Scemama et a. 2006; LePabic et al. 2007; Davis et al. 2008; Amores et al. 2004; Prince et al. 1998). Despite the differing expressions among the HoxA2b genes of teleosts in the rhombomeres they all show weak expression in PA2 except for $F$. heteroclitus, which has strong expression. This is the first instance of strong expression of HoxA2b in PA2 and also the first instance of definite and strong expression of both HoxA2 paralogs in PA2. Due to the differing expression patterns of HoxA2b and the strong conservation of HoxA2a expression we
furthered our study to look for evidence of selection in the enhancer sequences of these paralogs.

### 4.3 Mutation Rate Analysis of Hox A2 Loci

### 4.3.1 Phylogenetics of Hox A2 Loci

## Materials and Methods

To determine mutation rates among HoxA2 loci we first created phylogenetic trees of each locus. Nucleotide alignments were performed using the clustalW algorithm provided by MacVector® ${ }^{\circledR}$ 9.5.2. The alignments were corrected by eye so that all sequences started at the same position. 4 types of gene trees were constructed including neighbor-joining, maximum parsimony, maximum likelihood and Bayesian. Although neighbor-joining and maximum parsimony are considered antiquated there are some cases in which they have been shown to be more accurate (Landan and Graur 2007). For the more sophisticated tree building methods it was necessary to find an optimal model and prior inputs.

ModelTest v3.7 (Posada and Crandall 1998) was used to select the likelihood model for the nucleotide data. The model selected for the HoxA2 alignment by the hierarchical likelihood ratio tests (hLRT) (Gaut and Weir 1994) was HKY+G and by the Bayesian information criterion (BIC) (Raftery 1986a, 1986b) was HKY+G. We chose to use the HKY+G model which estimated the following parameters: Base frequencies; $\mathrm{A}=0.2333, \mathrm{C}=0.3466, \mathrm{G}=0.2580, \mathrm{~T}=0.1620 . \mathrm{Ti} / \mathrm{Tv}$ ratio of 1.4986 . Among site rate variation; proportion of invariable sites=0, Gamma distribution shape parameter=0.8828. The model selected for the HoxA2 enhancer alignment by the hierarchical likelihood ratio tests (hLRT) (Gaut and Weir 1994) was K80+G and by the Bayesian information criterion (BIC) (Raftery 1986a, 1986b) was K81+G. We chose to use the K80+G model, which estimated the following parameters: Base frequencies; equal frequencies. $\mathrm{Ti} / \mathrm{Tv}$
ratio of 0.9952 . Among site rate variation; proportion of invariable sites=0, Gamma distribution shape parameter $=1.0289$.

MrModelTest v2.2 (Nylander 2004) was used to select the likelihood model for the nucleotide data that could be implemented by MrBayes. The model selected for the HoxA2 alignment by hLRT, hLRT2, hLRT3 and hLRT4 was HKY+G. The model selected by AIC was HKY+G. We chose to use the HKY+G model which when implemented in MrBayes gives a prior state frequency of dirichlet $(1,1,1,1)$ and uses the basic model nst=2 with among site rate variation set to estimate rates based on a gamma shaped distribution. The model selected for the HoxA2 enhancer alignment by hLRT, hLRT2, hLRT3 and hLRT4 was K80+G. The model selected by AIC was HKY+G. We chose to use the K80+G model which when implemented in MrBayes gives a prior state frequency of fixed (equal) and uses the basic model nst=2 with among site rate variation set to estimate rates based on a gamma shaped distribution.

Neighbor Joining trees for the nucleotide alignments were constructed using PAUP* v4.0b10 (Swofford 2003). The HoxA2 and HoxA2 enhancer trees were constructed under the following assumptions; distances were calculated using the Tamura-Nei method (Tamura and Nei 1993), using parameters specified from models selected above. The objective function was set to minimum evolution and TBR was selected as the swapping algorithm. Trees were constructed using the Neighbor Joining method (Saitou and Nei 1987) and then 2000 bootstraps were conducted to assess node confidence. A consensus tree was created using majority rule with a $50 \%$ cutoff.

Maximum parsimony trees for nucleotide alignments were constructed using PAUP* v4.0b10 (Swofford 2003). All nucleotide trees were constructed under the
default settings using a heuristic search and node confidence was assessed with 2000 bootstraps. A consensus tree was constructed using majority rule with a $50 \%$ cutoff.

Maximum likelihood trees for the nucleotide data of both alignments were constructed using GARLI v0.951 (Zwickl 2006), which can be downloaded from www.bio.utexas.edu/faculty/antisense/garli/Garli.html. The starting tree was obtained using heuristic search under the likelihood optimality criterion in PAUP* v4.0b10 (Swofford 2003) with settings as specified by Modeltest (Posada and Crandall 1998, see Model Selection). The same settings were inputted into GARLI v0.951 (Zwickl 2006). Node confidence was assessed by using the bootstrap resampling method with 2,000 replications. The "number of generations without improving topology" setting was changed from 10,000 to 5,000 as suggested in the GARLI manual when doing bootstraps as it will shorten the run time without significantly affecting the results. A consensus tree was created using majority rule with a $50 \%$ cutoff in PAUP* v4.0b10 (Swofford 2003).

Bayesian trees for the nucleotide alignments were constructed using MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003) and the parallel version of MrBayes v3.1.2 (Altekar et al. 2004) under the settings specified by MrModeltest (see Model Selection). Two independent Markov Chain Monte Carlo analyses were run with the following settings: number of generations was set to $1,000,000$, sample frequency was taken every 1,000 steps, number of chains was set to 4 and the temperature was set 0.2 . 'Burnin' was assessed after the run using the sum parameters command. The 'burnin' for the nucleotide analysis was set to 1 for both datasets, which is equal to the first 1,000 steps or tree topologies. A majority rule consensus tree was created disregarding the 'burnin' trees using the sum trees command with a cut off of 0.50 posterior probability.

Phylogenetic analysis indicated that HoxA2a, HoxA2b, HoxA2a enhancer and HoxA2b enhancer sequences isolated from F. heteroclitus as well as northern studfish (Fundulus catenatus) and the two species that were later used in population level studies, black-striped topminnow (Fundulus notatus) and black-spotted topminnow (Fundulus olivaceus) were true paralogs and were the result of the same duplication as the rest of the teleosts' HoxA2 sequences, FSGD (Figure 17). Overall, gene trees appear to agree with accepted species phylogeny.

### 4.3.2 Mutation Rate Analysis

Gene trees and alignments were used as input data for a character state tree constructed using MacClade v4 (Maddison and Maddison 1989) for the 156 bp spanning NC2 to RE3 which also encompasses the entire RE4 and 5' half of NC3. From this analysis it is apparent that the ' $b$ ' paralog has acquired more changes than the ' $a$ ' paralog and at a seemingly steady rate (Figure 18). The changes in the 'a' paralog are concentrated at the base of the teleosts (35) prior to the rapid speciation with significantly ( $\mathrm{p}=0.00468$ ) fewer changes in all of the following internal and terminal branches (34 total) as compared to identical branches in the 'b' paralog (53 base and 122 other). The ' $b$ ' paralog however, has a continuous change in characters with no less than 23 on any branch. The Fundulus sp. 'b' paralog has acquired more changes than any other 'b' paralog terminal with at least 36 changes. This evidence of continued turnover in the ' $b$ ' paralog binding sites well into the Fundulus $s p$. lineage and lack thereof in the 'a' paralog is in contrast to the more conserved nature of the ' $b$ ' paralog sequence between the two in-group species of Fundulus $s p$. This is more apparent taking into consideration changes in highly conserved binding sites. When comparing only those sites that have one step in
the tree (those sites where all taxa except for one share the same state) the Fundulus $s p$. 'b' paralogs have at least twice as many steps as any other fish/paralog. This indicates that leading to the Fundulus $s p$. lineage changes in highly conserved nucleotides were at least twice as likely to become fixed then in any other fish lineage. This is further supported by the fact that changes within the Fundulus sp. group at the 'b' paralog enhancer are concentrated around functional sites. This same pattern can be seen in the HoxA2 coding sequences as well.

### 4.4 Population Genetic Study of Fundulus Hox A2 Enhancer Evolution

### 4.4.1 Specimen Acquisition

## Materials and Methods

To perform a population genetic study on Fundulus it was necessary to acquire wild Fundulus fish because no lab strains exist, we needed individuals from two sister species and lab strains are unlikely to maintain the variation that existed before they were institutionalized. For the purpose of the population genetic study we collected individuals from two sister species of killifish, black-striped topminnow and blackspotted topminnow from Mississippi River drainages in Illinois and Missouri with the help of Dr. David Duvernell of University of Southern Illinois-Edwardsville and Robert Lynch (lab member). F. olivaceus was collected from the west side of the Mississippi River (Missouri) from 3 separate locations comprising two separate river drainage systems. These species were chosen due to the glut of knowledge of their phylogeography, well studied relationship, location within the United States and availability of expert on-site help. From the $1^{\text {st }}$ drainage system we collected 93 individuals from one site, which was Rockford Beach at Big River at House Springs ( $38^{\circ}$ $25^{\prime} 20.27^{\prime \prime} \mathrm{N}, 90^{\circ} 35^{\prime} 23.58^{\prime \prime}$ W, from here on known as Rockford Beach). From the $2^{\text {nd }}$ drainage system we collected 29 individuals from two sites which were Little Piney Creek ( 5 individuals) at Highway 44 bridge at Jerome ( $37^{\circ} 55^{\prime} 04.68^{\prime \prime}$ N, $91^{\circ} 58^{\prime} 16.66 "$ W, from here on known as Little Piney) and Gasconade River (24 individuals) at Jerome ( $37^{\circ} 56^{\prime} 05.34^{\prime \prime} \mathrm{N}, 91^{\circ} 58^{\prime} 39.24^{\prime \prime} \mathrm{W}$, from here on known as Gasconade River). $F$. notatus was collected from the east side of the Mississippi River (Illinois) from 3 separate locations comprising two separate river drainage systems. From the $1^{\text {st }}$ drainage system
we collected 42 individuals from one site, which was Salt Creek at Highway 40 bridge east of Effingham ( $39^{\circ} 07^{\prime} 15.49^{\prime \prime} \mathrm{N}, 88^{\circ} 31^{\prime} 24.00^{\prime \prime} \mathrm{W}$, from here on known as Salt Creek). From the $2^{\text {nd }}$ drainage system we collected 64 individuals from two separate locations which were Kaskaskia River at Highway 2700N north of Vandalia ( $39^{\circ} 07^{\prime}$ $53.79^{\prime \prime} \mathrm{N}, 90^{\circ} 35^{\prime} 23.58^{\prime \prime}$ W, from here on known as Kaskaskia River) and Big Creek at Highway 2100E and 2600 N intersection north of Saint Elmo ( $39^{\circ} 06^{\prime} 57.32^{\prime \prime} \mathrm{N}, 88^{\circ} 51^{\prime}$ 42.81 " W, from here on known as Big Creek). Approximately 12 individuals of an outgroup taxa northern studfish were also collected at Little Piney. The fish were collected on September $16^{\text {th }}$ and $17^{\text {th }}, 2006$. Collection of individuals was done using seines and hand-nets. We preserved individuals on-site in 95\% ethanol. Individuals were later placed into separate, numbered storage bottles in $95 \%$ ethanol.

We performed DNA isolation of individuals using fin clips under standard tissue preparation protocols using the AquaPure Genomic DNA Tissue Kit by Bio-Rad.

### 4.4.2 Gene Isolation

## Materials and Methods

We isolated HoxA9b, A3a, A2a, A2b and B2a genes; HoxA2a and HoxA2b r3/5 enhancers as well as phosphoglycerate kinase 1 (PGK1) from $F$. notatus and $F$. olivaceus using PCR protocols as described in section 2.2.1 with the following modifications: HoxA9b was isolated with A9b forward (5'-CTTTGGAGACGCACACWCC-3') and A9b reverse (5'-TTCTTCATCTTCATYCTGCGG-3') primers at an annealing temperature of 57 C ; HoxA3a was isolated with A3a forward (5'-ARTACAARAAGGATCAGAAAGG-3') and A3a reverse (5'-TTRCCCATTGTGATTGCTCC-3') primers at an annealing temperature of 57 C;

HoxA2a was isolated with A2a forward (5'-GAATTCGARCGAGAGAGCG-3') and A2a reverse ( $5^{\prime}$-ACRGGTCCGTTKGAGATGG-3') primers at an annealing temperature of 56 C ; Hox B 2 a was isolated with B 2 a forward (5'-

CATTTCAAACTTCATCAATCAAGG-3') and B2a reverse (5'-
CTCYTTCATCCAKGGRAACTC-3') primers at an annealing temperature of 58 C ;
PGK1 was isolated with PGK1 intron 7 forward (5'-
GCRAAGGTGAAAGATAAGATYCAGC-3') and PGK1 intron 7 reverse (5'-TCTCSGCTTTGGCCATCAGG-3') primers at an annealing temperature of 62 C .

HoxA9b and HoxA3a were subjected to the same phylogenetic analysis as $H o x A 2 \mathrm{a}$ and HoxA2b to determine their paralog status with the following gene specific input data: Regarding ModelTest v3.7 (Posada and Crandall 1998), the model selected for the HoxA3a alignment by the hierarchical likelihood ratio tests (hLRT) (Gaut and Weir 1994) was HKY+G (Hasegawa et al. 1985) and by the Bayesian information criterion (BIC) (Raftery 1986a, 1986b) was HKY+G. We chose to use the HKY+G model which estimated the following parameters: Base frequencies; $\mathrm{A}=0.2236$, $\mathrm{C}=0.3432, \mathrm{G}=0.2357, \mathrm{~T}=0.1975$. $\mathrm{Ti} / \mathrm{Tv}$ ratio of 1.4676 . Among site rate variation; proportion of invariable sites $=0$, Gamma distribution shape parameter $=0.6010$. The model selected for the HoxA9b alignment by the hierarchical likelihood ratio tests (hLRT) (Gaut and Weir 1994) was HKY+I+G and by the Bayesian information criterion (BIC) (Raftery 1986a, 1986b) was HKY+I+G. We chose to use the HKY+I+G model which estimated the following parameters: Base frequencies; $\mathrm{A}=0.2923, \mathrm{C}=0.2753$, $\mathrm{G}=0.2391, \mathrm{~T}=0.1933 \mathrm{Ti} / \mathrm{Tv}$ ratio of 1.4986 . Among site rate variation; proportion of invariable sites=0.2648, Gamma distribution shape parameter=1.7812. Regarding

MrModelTest v2.2 (Nylander 2004), the model selected for the HoxA3a alignment by hLRT, hLRT2, and hLRT4 was HKY+G, hLRT3 was GTR+G (Lanave et al. 1984). The model selected by AIC was GTR+G. We chose to use the HKY+G model which when implemented in MrBayes gives a prior state frequency of Dirichlet $(1,1,1,1)$ and uses the basic model nst=2 with among site rate variation set to estimate rates based on a gamma shaped distribution. The model selected for the HoxA9b alignment by hLRT2, hLRT3, and hLRT4 was HKY $+\mathrm{I}+\mathrm{G}$, hLRT was GTR $+\mathrm{I}+\mathrm{G}$. The model selected by AIC was HKY $+\mathrm{I}+\mathrm{G}$. We chose to use the HKY $+\mathrm{I}+\mathrm{G}$ model which when implemented in MrBayes gives a prior state frequency of dirichlet $(1,1,1,1)$ and uses the basic model nst=2 with among site rate variation set to estimate rates based on a gamma shaped distribution and proportion of invariable sites.

To empirically link the HoxA2 enhancers to the genes they regulate the entire intergenic region of $H o x A 3 a$ to HoxA2a was isolated in F. catenatus. We used Takara LA Taq due to its ability to amplify longer DNA fragments with the following volumes and concentrations of reagents: 5.0 ul 10 X buffer, $4.0 \mathrm{ul} \mathrm{MgCl}_{2}(25 \mathrm{mM}), 4.0 \mathrm{ul}$ dNTP ( 10 mM ), 1.0 ul each of primers ( $25 \mathrm{pmol} / \mathrm{ul}$ ), $1.0 \mathrm{ul} \mathrm{gDNA}(250 \mathrm{ng} / \mathrm{ul})$, and 1.0 ul LA Taq ( $5 \mathrm{U} / \mathrm{ul}$ ). We conducted the PCR reaction under the following conditions: 95 C for $2^{\prime}, 30$ cycles of ( 95 C for 30 ', 55 C for $1^{\prime}, 68 \mathrm{C}$ for $4^{\prime}$ ), 68 C for $5^{\prime}$ and 10 C hold. Due to unknown product size and multiple bands we performed a southern blot and hybridization.

We performed the southern blot procedure by transferring the PCR reaction from a $1 \%$ agarose gel to Amersham pharmacia biotech's Hybond-N+ nylon membrane. We performed transfer of DNA from the agarose gel to the nylon membrane as follows:

Soaked gel in southern denaturation solution ( $0.2 \mathrm{M} \mathrm{NaOH}, 0.6 \mathrm{M} \mathrm{NaCl}$ ) for $30^{\prime}$ on a rocker. Soaked gel in neutralization solution for 30' on a rocker. Placed two sheets of Whatman's paper cut to size of gel in a glass dish, filled to top of Whatman's paper with 20X SSC (3.0 M NaCl, 0.3 M sodium citrate), place gel on top of paper, place Hybond$\mathrm{N}+$ membrane on gel, place two more pieces of Whatman's paper followed by 5 inches of paper towels and a $\sim 1 \mathrm{lb}$ weight, add 20X SSC 1 hr later, wrapped entire assembly in plastic wrap and let sit overnight. We cut all paper and membrane to the exact size of the gel. We then let the membrane dry and pre-hybridized overnight in 10 ml hybridization solution (5X SSC, $0.1 \% v / v \mathrm{~N}$-Lauroylsarcosine, $0.2 \% v / v$ sodium dodecyl sulfate, $1 \%$ $w / v$ blocking solution (Roche Diagnostics Blocking reagent)) at 65 C in rotisserie incubator along with a positive filter consisting of HoxA3a fragment on the nylon membrane. We prepared the positive filter by placing $1 \mathrm{ul}(100 \mathrm{ng} / \mathrm{ul})$ of fragment on a nylon membrane, drying, then floating in southern denaturation solution for 3 ' followed by floating in neutralization solution for $3^{\prime}$.

We used the $451 \mathrm{bp} H o x A 3$ a fragment isolated from $F$. catenatus genomic DNA as a probe. We DIG (Digoxigenin)-labeled the HoxA3a fragment using Roche Diagnostics DIG DNA labeling kit (11175033910). The DIG labeling reaction includes boiling $800 \mathrm{ng}(15 \mathrm{ul})$ of the HoxA3a fragment in water for $10^{\prime}$, placing on ice $\left(\mathrm{H}_{2} \mathrm{O}\right)$ for 10 ', briefly spinning down, then adding 2 ul of the hexanucleotides, 2 ul dNTP labeling mix, and 1ul Klenow enzyme provided in Roche kit. We then placed the labeling reaction at 37 C for 20 hrs .

After pre-hybridization and probe labeling we re-boiled the probe for $10^{\prime}$ and chilled on ice for $10^{\prime}$. We then added the 20 ul of probe to 10 ml of hybridization
solution and hybridized membranes overnight in rotisserie incubator. We then washed the membranes briefly in 2 X SSC followed by 2 washes in 2 X SSC, $0.1 \% \mathrm{v} / \mathrm{v}$ SDS for $20^{\prime}$ at room temperature on a rocker followed by 2 washes in 0.5 X SSC, $0.1 \% \mathrm{v} / \mathrm{v} \mathrm{SDS}$ for 20' at 65 C in rotisserie incubator. We then rinsed filter in diluent buffer ( 300 mM $\mathrm{NaCl}, 0.1 \mathrm{M}$ Tris pH 8.0 ). We followed this by a "blocking" step by placing membranes in 40 ml of diluent buffer, $4 \mathrm{ml} \mathrm{10} \mathrm{\%} \mathrm{blocking} \mathrm{solution} \mathrm{for} 1 \mathrm{hr}$ on a rocker then an "antiblocking" step by placing membranes in 30 ml of diluent buffer, $4 \mathrm{ml} 10 \%$ blocking solution, 3 uL of Roche Diagnostics Anti-DIG-AP Fab fragments ( $0.75 \mathrm{U} / \mathrm{ul}$ ). We followed this with 3 washes in a tween solution ( 150 ul tween $20,50 \mathrm{ml}$ diluent buffer) for 10 ' on a rocker. We followed this with washing in 50 ml of detection buffer (100 mM Tris $\mathrm{pH} 9.5,100 \mathrm{mM} \mathrm{NaCl}$ ) for $5^{\prime}$ ' on a rocker. We then prepared the membranes for exposure to Kodak Biomax light film. We prepared the membranes for exposure by adding Roche Diagnostics CDP-star to their surface. We then exposed the membranes to the film for $10^{\prime}$ and then the film was developed on a Kodak developer.

Following identification of correct band $(\sim 6.5 \mathrm{~kb})$ by southern hybridization we followed the protocol explained above to sequence the fragment. We had to create 3 sets of species-specific nested primers to sequence the entire fragment We used Takara LA Taq with the following volumes and concentrations of reagents: 5.0 ul 10 X buffer, 4.0 ul $\mathrm{MgCl}_{2}(25 \mathrm{mM}), 4.0 \mathrm{ul} \mathrm{dNTP}(10 \mathrm{mM}), 1.0 \mathrm{ul}$ each of primers ( $25 \mathrm{pmol} / \mathrm{ul}$ ), 1.0 ul gDNA ( $250 \mathrm{ng} / \mathrm{ul}$ ), and $1.0 \mathrm{ul} \mathrm{LA} \mathrm{Taq} \mathrm{( } 5 \mathrm{U} / \mathrm{ul}$ ). We conducted the PCR reaction under the following conditions: 95 C for $2^{\prime}, 30$ cycles of ( 95 C for 30 ', 55 C for $1^{\prime}, 68 \mathrm{C}$ for $4^{\prime}$ ), 68 C for $5^{\prime}$ and 10 C hold.

### 4.4.3 Population Level Gene Sequencing

## Materials and Methods

HoxA2a enhancer, HoxA2b enhancer and a shorter fragment of PGK1 intron 7 were isolated from $\sim 100$ individuals of the $F$. olivaceus populations. These were done with Finnzymes Phusion Taq due to its 50 times greater accuracy over AB amplitaq. Phusion Taq PCR reactions, volumes and concentrations were as follows: 10.0 ul 5 X HF buffer, 4.0-7.0 ul MgCl $2(25 \mathrm{mM}), 1.0 \mathrm{ul} \mathrm{dNTP}(10 \mathrm{mM}), 1.0 \mathrm{ul}$ each of primers ( 25 $\mathrm{pmol} / \mathrm{ul}), 1.0 \mathrm{ul}$ gDNA ( $250 \mathrm{ng} / \mathrm{ul}$ ), 0.5 ul Phusion Taq ( $2 \mathrm{U} / \mathrm{ul}$ ) and sterile water to 50 ul . The PCR reaction for Phusion Taq was conducted under the following conditions: 98 C for 1', 30 cycles of ( 98 C for 30 ", annealing temperature (see table 2 ) for 30 ", 72 C for $30^{\prime \prime}$ ), 72 C for 5' and 10 C hold. All PCR reactions were carried out in an Eppendorf Mastercycler® ep thermocycler. Following PCR, the PCR products were sent directly from the gel purification step to be sequenced. Approximately 70 ng of purified PCR product was sent. This method of direct PCR sequencing reduces the effect of base pair mispairing that can occur with DNA polymerases due to the fragments with the mispairing representing a minority of the fragments in the sequencing reaction.

### 4.4.4 Tests of Selection

We conducted both interspecies and intraspecies tests of selection on the Fundulus sp. group (Table 1). For interspecies comparisons we calculated the $\mathrm{Ka} / \mathrm{Ks}$ ratio (Nei and Gojobori 1986). This test was performed on all coding regions that were sequenced (Table 1). These results were directly compared with the $\mathrm{Kb} / \mathrm{Ki}$ ratio (Hahn 2007, see materials and methods), which was implemented for the non-coding HoxA2 enhancers. These ratios are an indicator of selective forces acting at these loci. A ratio of
one is an indicator that there is no selection and the nucleotides are neutrally evolving. More than one denotes positive selection and less than one denotes negative or purifying selection. The majority of loci indicate negative or purifying selection.

Since the Hox loci are master regulatory genes found throughout the animal kingdom, selection to maintain nucleotide sequence and thus protein function is likely. The non-Hox locus PGK1 also appears to be under purifying selection. The lone exception to the neutral theory of evolution is the comparison between in-group species F. olivaceus and $F$. notatus at the HoxA2b enhancer. This comparison yields an exceptionally high $\mathrm{Kb} / \mathrm{Ki}$ ratio of $7.78(\mathrm{p}=0.03)$ indicating strong positive selection as described in Hahn et al. (2004). The positive selection is isolated to this node as the $\mathrm{Kb} / \mathrm{Ki}$ ratios between either of these two sister species and the out-group species, $F$. catenatus, is close to and not significantly different than 1 . Further indication of positive selection can be noted by comparing the non-coding/non-binding percent difference for each locus, which is an indicator of neutral evolution at each site.

As seen in table 1 the two sister species, $F$. olivaceus and $F$. notatus have a lower percent difference than either do to the out-group, F. catenatus, most likely due to the divergence dates of these species. The more notable difference is the divergence seen between sister species when comparing the two HoxA2 enhancers; HoxA2b enhancer $0.48 \%$ and HoxA2a enhancer $2.02 \%$. Compared to the HoxAb cluster genes which are $2.63 \%$ and $2.95 \%$ and the HoxAa cluster genes which are $0.66 \%$ and $0.90 \%$ there seems to be an inverse relationship between enhancer and gene neutral mutation rates at the ' $a$ ' and 'b' paralog loci (Figure 19). This inverse relationship is not seen however in the comparison between either of the sister species to the out-group species. At this level the
$H o x A 2 a$ enhancer is more conserved at $4.03 \%$ for both comparisons than is the HoxA2b enhancer, $4.76 \%$ and $5.24 \%$.

Also, between the sister species no other locus has more than 1 fixed difference in the functional region whereas the HoxA2b enhancer has 4. This evidence that the neutral mutation rate was slowed at the non-binding sites in the HoxA2b enhancer between $F$. olivaceus and $F$. notatus strengthens the claim that nucleotides at binding sites were preferentially fixed in the population (positive selection) at this enhancer.

Following the method used by Hughes and Nei 1988, we also applied the $\mathrm{Kb} / \mathrm{Ki}$ ratio test to the $F$. olivaceus within species data. The HoxA2a enhancer had a $\mathrm{Kb} / \mathrm{Ki}$ ratio of $0.00(\mathrm{p}=0.59)$ indicating purifying selection. The HoxA2b had a Kb/Ki ratio of 1.94 ( $\mathrm{p}=0.63$ ) suggesting balancing selection although neither test was statistically significant. The neutral polymorphism at these two sites was less than the non-Hox locus with the HoxA2a enhancer at $0.76 \%$, the HoxA2b enhancer at $0.48 \%$ and intron 7 of the PGK1 gene at $2.4 \%$. We also performed conventional intraspecies tests of selection using the software programs Neutrality Test v1.1 (Li et al. 2003) and DnaSP v4.0 (Rozas et al. 2003) on the HoxA2b enhancer. These test were not performed on the HoxA2a enhancer because alleles could not be confidently assigned to individuals. These programs gave a Tajima's D score of 1.681 and 1.13474 standard deviations from the mean for the $H o x \mathrm{~A} 2 \mathrm{~b}$ enhancer indicating an excess of high frequency polymorphisms that is interpreted as balancing selection, which was shown to not be significant. Fu and Li's D and F statistics were 0.68090 and 0.96107 respectively without an out-group and changed less than 0.001 when an out-group was included. Again these tests did not have significance ( $p>0.05$ ) but fell in the direction of balancing selection. In order to take
advantage of the multiple loci and species an HKA test was performed (results not shown) but the results again were not statistically significant. It appears that within species the HoxA2b enhancer is evolving under no or slightly balancing selection but between $F$. olivaceus and $F$. notatus there was strong positive selection.

We also performed a Hardy-Weinberg (H-W) equilibrium test on the HoxA2b enhancer of the F. olivaceus population to show that our sample was a random set of the population. This is an important step as many tests of selection assume random sampling. The $F$. olivaceus population has two distinct sub-populations gathered from three sites. The first sub-population was gathered from two sites along the same river drainage that are named Little Piney and Gasconade River (see materials and methods). The second sub-population was gathered from one site and is named Rockford Beach. The Little Piney/Gasconade River sub-population showed no allele variants at the HoxA2b enhancer. The Rockford Beach sub-population had two allele variants that were distinct from the one found in Little Piney/Gasconade River for a total of three alleles. These three alleles were found to be in Hardy-Weinberg equilibrium within the Rockford Beach sub-population ( $\mathrm{p}=0.62$ ). This verified that our sample of the population was not biased which could alter tests of selection that assume random mating.

Given the data we collected and the lack of information about the divergence dates between our two sister species we performed a divergence date estimation. The nuclear gene estimate of the divergence date between $F$. olivaceus and $F$. notatus was calculated by calculating the distance between 7 homologous regions (HoxB2a exon 1,HoxA9b exon 1, 2 and intron, HoxA2b exon 1,HoxA3a exon 2, and HoxA2a exon 1,HoxA2a enhancer and HoxA2b enhancer) totaling 1,395 bp. Divergence date estimations for the
three Fundulus sp. involved in this study were performed based on the \% difference of the sequences accumulated. This was then compared to two pufferfish species (Tetraodon nigroviridis and Takifugu rubripes) with a known divergence date to estimate divergence date for the Fundulus $s p$. assuming an equal rate of mutation for these two Acanthomorpha clades. Overall F. olivaceus and F. notatus appear to have diverged around $\sim 4.76$ mya. This is the first nuclear data used to estimate divergence dates for these two species to our knowledge and agrees with the date derived from mitochondrial data $\sim 2.75$ mya (personal communication, Brian Kreiser).

### 4.4.5 In-silico Detection of Upstream Binding Factors

To better understand the expression patterns and evidence of selection it is necessary to know the nature of the enhancer. The HoxA2 R3/5 enhancer consists of 7 modules. RE2-5 help code for expression in r3 and 5 and NC1, 2 and 3 code for expression in neural crest cells derived from r4 and their derivative (PA2) (Nonchev et al. 1996; Maconochie et al. 2001; Tumpel et al. 2006). Three main transcription factors are thought to control HoxA2 expression via the R3/R5 enhancer: Krox-20, which controls expression in r3, 5 (Nonchev et al. 1996); Mafb, which control expression in r5 (Prince et al. 1998); and AP-2, which controls expression in PA2 (Maconochie et al. 1999). Since the domains of the enhancer that code for both r3 and 5 expression (RE4) and neural crest cell expression (NC2) overlap in the area of strong positive selection it is possible that we are seeing strong purifying selection elsewhere in the enhancer (RE2, 3, 5) (see divergence comparisons above) to maintain rhombomere expression in the presence of strong positive selection (RE4/NC2) to increase expression in the neural crest cells and their derivatives. This is supported by Tumpel et al. 2006, who showed RE4 is $32 \%$
efficient at driving R3/5 expression and RE3 and RE5 were $67 \%$ and $33 \%$ efficient, respectively with RE1 and RE2 contributing negligible amounts. So maintaining strong purifying selection in RE3 and RE5 could compensate for the loss of expression in RE4 due to its overlap with the strong positive selection to modify NC2. It is also possible that upstream factors that normally bind to RE4 to drive expression in r3 and 5 have coevolved with the nucleotide changes to maintain expression. A third possibility that compensatory mutations elsewhere in the enhancer are compensating for the loss of specificity at RE4 seems less likely since the rest of the enhancer is so well conserved. To help differentiate between the three possible scenarios TFBS prediction program JASPAR was used to analyze possible TFBS changes between $F$. notatus and $F$. olivaceus at the changes within RE4/NC2 (Sandelin et al. 2004; Figure 20). All 4 fixed differences resulted in different TFBS profiles and this region is flanked by two RUSH$1 \alpha$ sites ( $5^{\prime}$ site and $3^{\prime}$ site) that are present in all 4 species (Figure 20). Both 5' and 3' RUSH- $1 \alpha$ sites have shifted toward each other by 2 nucleotides in the two sister species due to two mutations shared by these species that are not present in either $F$. heteroclitus or $F$. catenatus (Figure 20). Despite these mutations both sites have maintained similar scores in the JASPAR database. RUSH-1 $\alpha$ is a SWI/SNF-related matrix-associated protein, which possesses nucleosome remodeling activity and may be necessary for basal transcription of target genes (Hewetson et al. 2002). RUSH transcription factors also contain a RING finger domain at the c-terminal end that plays a role in protein-protein interactions (Mansharamani et al. 2001). RUSH-1 $\alpha$ binding sites share sequence similarity with FOXL1 and FOXC1 binding sites.

FOXL1 and FOXC1 are forkhead family transcription factors that play roles in anterior segment development (Hacker et al. 1995). All four species maintain either 1 FOXC1 or FOXL1 at both 5' and 3' sites but $F$. notatus has binding sites for both forkhead family member at both 5' and 3' sites (Figure 20). The 3' site also has affinity for Ultrabithorax (Ubx). Ubx is orthologous to HoxA7 in vertebrates and although there is no evidence that HoxA7 regulates hindbrain development it is worth noting that $F$. olivaceus loses this Ubx TFBS at the 3 ' site but gains it back at the 5 ' site. Both $F$. olivaceus and $F$. notatus independently gain an HMG-1 binding site. HMG-1 helps regulate transcription, differentiation and DNA repair (Bianchi and Beltrame 2000; Mitsouras et al. 2002). HMG-1 contains two DNA binding sites along with a C-terminal protein interaction domain that has been shown to interact with Hox proteins and enhance their DNA binding and transcriptional activation (Zappavigna et al. 1996). The Ubx and HMG-1 site are of importance due to the discovery that Hox genes often auto and crossregulate (Hafen et al. 1984; Kuziora and McGinnis 1988). The most pertinent TFBS change occurs in $F$. notatus midway between the $5^{\prime}$ and $3^{\prime}$ site. At this site all species except for $F$. notatus maintain an Mafb binding site. Mafb also known as kreisler (mouse) or valentino (zebrafish) is expressed in the developing hindbrain and is essential for r5, 6 development and patterning (Prince et al. 1998b; Sadl et al. 2003). The 1 fixed difference between $F$. olivaceus and $F$. notatus that lies outside of any known functional element causes $F$. notatus to have an Mafb binding site that the other three Fundulus $s p$. do not. From this data there is evidence of both positive selection to gain binding sites as well as compensatory mutations to maintain them.

If we subject all other vertebrate HoxA2 enhancer sequences to the same type of analysis for Mafb binding sites as well as AP-2 and Krox20 binding sites a unique pattern emerges. Comparing binding sites of all three major R3/R5 enhancer transcription factors across vertebrate phylogeny allows us to see that RE3 is the most well conserved response element, whereas RE4 appears to be less conserved (Figure 21). This is in agreement with the idea that RE3 is responsible for a more basal expression whereas RE4 can act as a "fine-tuning" element. Tumpel et al. (2006) also showed this in their swap assays where RE3 recapitulated $67 \%$ whereas RE4 recapitulated $32 \%$ of the normal expression. Despite RE3 being the more conserved region the Fundulus sp. appear to be more variable than other species, specifically in the loss of highly conserved AP-2 binding sites. The two sister species have both lost an AP-2 binding site that is conserved among all enhancers except the HoxA2a enhancers, including the two other members of the Fundulus sp. group. It also appears that all of the Fundulus sp. have lost another AP2 binding site in RE3 that is shared among all ray-finned fish including both 'a' and ' $b$ ' paralogs and also with chicken, frog and coelacanth. The region between the Krox-20 binding site and RE4 has been shown to be non-functional in both deletion analysis and swap assays (Maconochie et al. 2001; Tumpel et al. 2006). In this region a mutation in $F$. notatus adds back an Mafb binding site that no other Fundulus sp. member has. Also, the entire Fundulus sp. group lacks any AP-2 binding sites. This is possibly due to the ability for spurious binding sites to 'sequester' transcriptions factors away from the functional binding sites since the Fundulus sp. group specifically has lost 2 well-conserved AP-2 binding sites in RE3.

### 4.5 Discussion

$H o x \mathrm{~A} 2$ is the most anteriorly expressed Hox gene in anterior-posterior patterning. The expression pattern of this gene has been shown for many vertebrates including birds, mammals and fish. In ray-finned fishes the expression pattern is made more complex due to the retention of two paralogs following the FSGD. Much work has gone into discovering the CREs responsible for driving expression of HoxA2 in the developing nervous system and derived structures. From this work a detailed map of the CREs has emerged. In this study we set forth to gather data that would help shine light on the evolution of one of these particular CREs. From this study we have discovered that the r3/r5 enhancer of the HoxA2 gene has undergone different rates of mutation throughout gnathostome evolution. More specifically, the retention of HoxA2 paralogs in the teleosts resulted in asymmetrical evolution. The HoxA2a r3/r5 enhancer appears to have undergone an accelerated rate of mutation fixation after the FSGD but prior to the Ostariophysii/Acanthomorpha species radiations. In contrast, the HoxA2b r3/r5 enhancer appears to have maintained an accelerated rate of mutation fixation prior to and throughout the teleost species radiation. The highest $\mathrm{r} 3 / \mathrm{r} 5$ enhancer mutation rate is maintained in the HoxA2b locus of the Fundulus sp. complex. Classification of these mutation fixations indicates that the mutations in TFBS were preferentially retained. Evolutionary maintenance of the $\mathrm{r} 3 / \mathrm{r} 5$ enhancers along with support from earlier expression assays indicates there are certain elements that may be more important to basal expression whereas other elements may act as 'fine-tuning' mechanisms. The Fundulus sp. complex is again unique with more loss of TFBSs in the basal elements than any other species/group.

In an effort to discover why/how the Fundulus sp. complex has so much nucleotide turnover in the otherwise more conserved and seemingly more basal regions of the $\mathrm{r} 3 / \mathrm{r} 5$ enhancer we performed micro-evolutionary tests. From these test we discovered support for evidence of strong positive selection occurring within this group between two closely related sister species. More specifically we saw 4 fixed differences within 38 base pairs in the RE4/NC2 region. TFBS analysis indicates these changes resulted in the two sister species each independently gaining the same Hox protein-binding partner. The loss of a Hox protein-binding site was regained in one species but the loss of the TFBS for Mafb (a TF necessary for HoxA2 expression in r5) in the other species was not regained. This shows that turnover of CREs at the macro-evolutionary level that appear to be due to relaxation of constraint and probable neutral evolution may actually be driven at least in part by strong positive selection at the micro-evolutionary level. This reiterates the need for more research into the evolutionary mechanisms that are shaping our CREs rather than research aimed at detecting the results of those mechanisms. Emphasis on the level at which evolution actually occurs may help shed a brighter light on how non-coding DNA contributes to adaptation and ultimately lead us to a more accurate and useful genetic code for regulatory DNA.

If the selective pressure is indeed due to the need to increase PA2 expression of HoxA2b, then what might be the possible phenotypes on which selection is acting? $H o x \mathrm{~A} 2$ has been shown to directly regulate bone morphogenetic protein 4 (BMP4; Smith et al. 2009). BMP4 is expressed in the lower oral jaw (LOJ) and is directly associated with altering the ratio between flexibility and bite force of the oral jaw (Albertson et al. 2005).

Liem's Hypothesis contains the idea, that since the adaptation of posterior pharyngeal arches into a pharyngeal jaw, that oral and pharyngeal jaws have become uncoupled and may evolve independently (Liem 1973). While we are not testing this hypothesis in this study it does help explain how such adaptations can occur without detriment to the species and how such a variation in HoxA2 expression could be tolerated within the teleosts.

Fundulus olivaceus and F. notatus have been well studied in their physical characteristics and habitat distribution (Petifils 1986). The only defined distinguishing characteristic between the two species is the presence of dark regular spots above the lateral line in F. olivaceus (Thomerson 1966). A possible explanation for the phenotypic difference that may be driving the positive selection for altered expression of HoxA2b in PA2 has to do with courtship. F. olivaceus males have been seen defending a $3 \mathrm{~m}^{2}$ territory from other males (Harper 1992). During this defense the male will flare its opercles and gular area when a female and another male are present (Baugh 1981). This behavior is not seen in $F$. notatus. It is possible that differing PA2 development could lead to the ability/inability to flare this area. Albertson et al. (2005) state the lever mechanism that is directly correlated with BMP4 expression is reliant on the ligament attaching the lower jaw to the opercle. QTL mapping in cichlids has also shown a correlation between directional selection at the genetic level and phenotypic effects in the LOJ (Albertson et al. 2003).

Other possible explanations include bite speed/force or digging ability for egg depositing. F. olivaceus and F. notatus both feed at the same trophic level on the same food stuff so the need for bite speed/force differences is unlikely (Thomerson and

Wooldrige 1970). The two sister species do prefer habitats with different floor surfaces although neither bury their eggs. Since HoxA2 is both partially responsible for the musculature and innervation of the LOJ several other phenotypes may also be affected.

In conclusion, we have found evidence of positive selection followed by balancing selection acting at the microevolutionary level in an enhancer that is differentially driving expression between two paralogs at the macroevolutionary level. Coincident with this selection is evidence of compensatory mutations. To our knowledge, this is the first study to research the evolutionary forces acting on a Hox cisregulatory element within and between sister species.

## Chapter 5 - Independent Evolutionary Mechanisms of Teleosts

## Uniqueness

### 5.1 Introduction

### 5.1.1 Transposable Elements Role in Genomic Evolution

Transposable elements (TE) were discovered by McClintock (1956) and are endogenous sequences of DNA that have the ability to transpose from place to place within the genome. There are two main classes of transposable elements. Class I transposons (a.ka. retrotransposons) transpose by the copy and paste method. First RNA is transcribed and then reverse transcribed by a reverse transcriptase encoded for by the element itself. Finally, the new DNA sequence is integrated back into the genome by an integrase also coded for by the element itself. Class II transposons (a.k.a. DNA transposons) transpose by the cut and paste method. A transposase protein coded for by the element itself excises the element, or any other element that contains the correct repeat sequences, from the genome and integrates it somewhere else in the genome.

Class II but not class I transposons have been found to be positively correlated with areas of high recombination within the genome (Duret et al. 2000). This association is theorized to be a cause-effect relationship rather than an exploitation of the endogenous double stranded DNA repair process, but not all class II transposons seem to have this ability. In a study done on class II transposable elements in C. elegans, Tc1 transposon family members were found in areas of high recombination whereas closely related Tc 3 and Tc 5 transposons were not (Rizzon et al. 2003).

The introduction of transposable elements near a gene can interrupt or in some cases introduce cis-regulatory sequences (McClintock 1956). TEs can also become exons
or introduce alternate splice sites within a gene (Kim et al. 2006; Huh et al. 2009). TEs can also contribute to the overall size of a genome. In some cases TEs compose nearly $50 \%$ or more of the entire genome (Lander et al. 2001). Because of the ability to affect genetic structure at both the gene and genome level, TEs are considered a major force in genomic evolution. Because TEs have the ability to reorganize, lengthen intergenic regions, as well as create general havoc to gene sequences it has been theorized that they would be preferentially excluded from well-organized and conserved gene clusters such as the Hox gene cluster (Fried et al. 2004).

### 5.1.2 Tc1 Transposons

As mentioned in the previous section Tc 1 transposons have been found to associate with areas of high recombination (Duret et al. 2000). Despite this we found them to be prevalent in ray-finned fishes Hox clusters. For this reason we pursued several methods aimed at detecting their possible role in Hox cluster evolution that relies on a basic understanding of their mechanism of action.

The Tc1 transposable element was originally found in a species of worm, Caenorhabditis elegans (Emmons et al. 1983, X01005.1). This Tc1 was found to be a high copy number transposable element with precise excision from the DNA sequence in which it was residing (Emmons et al. 1983). It was later discovered that Tc1 not only had sites of precise excision but also sites of imprecise excision resulting in the element leaving remnants of its own insertion or taking small sections of DNA from its insertion site (Ruan and Emmons 1987). The Tc1 transposable element also differs not only in its excision precision but also in its excision prowess i.e., the rate at which it excises or inserts. This difference in excision rate is apparent when comparing germ line excision
rate to the rate of excisions in somatic tissue. Excisions occur more than 1,000 times more often in somatic than in germ line tissue (Eide and Anderson 1988). This is important because the number of excisions has a direct effect on the mutagenic ability of the element, also only germ line "jumps" can be seen in the next generation. Earlier in the same decade Eide and Anderson (1985) found that Tc1 transposition was strain specific. This shows that the response to Tc1 differs between cell types as well as between individuals within the same species.

The structure of the Tc1 transposable element and the Tc1 transposase and their interaction were discovered not long after the Tc 1 sequence itself was obtained. Tc 1 is known to be a 1,610 base pair transposable element with 54 base pair terminal inverted repeats (Liao et al. 1983; Figure 23). There is one continuous open reading frame that codes for a 343 amino acid transposase (Vos et al. 1993). Structurally Tc1 transposase contains a bipartite DNA binding domain. The first domain is from amino acids 1-68 and the second domain is from amino acids 68-142 (Vos and Plasterk 1994). There is also the catalytic domain, characterized by its DDE (Aspartate, Aspartate, Glutamate) motif at positions 157, 247 and 282 respectively (Doak et al. 1994). The bipartite DNA binding domain of the Tc 1 transposase binds the Tc 1 transposable element by first binding to nucleotides 12-26, with the amino-terminal domain and then binding nucleotides 7-13 with the carboxy-terminal domain, if and only if the amino-terminal domain binds first (Riddle et al. 1997).

Upon excision the Tc1 transposable element inserts preferentially nearby and in some cases to specific sequences (Luo et al. 1998). In the case of Tc 1, that integration site has been shown to be a TA/AT palindrome sequence (Rosenzweig et al. 1983b). A
preference has also been shown for the TA palindrome to be flanked by specific nucleotides. The consensus sequence for Tc 1 integration is GARATATGT (the R represents A/G) (Mori et al. 1988).

In order for the transposable element to insert into the target sequence it must bring its 5 ' and $3^{\prime}$ ends into close proximity. This can be accomplished by forming an intermediate loop, as some introns excised from mRNA do. This is known to occur in the Tc1 excision reaction (Rose and Snutch 1984). Proteins from the host cell can also be involved in Tc1 transposition. Since there are differences in the "jumping" abilities of Tc 1 transposable elements in germ line and somatic tissue, it is fair to assume that different cell types differentially regulate its transposition. Shortly after Tc1 was discovered, it was shown that Tc 1 elements in 5 different loci on different chromosomes were more active in the somatic tissue opposed to the germ line tissue. The study also showed that this was caused by tissue-specific factors that either activated or suppressed the transposition (Emmons et al. 1986). Although there may be factors that help suppress or up-regulate transposition, they are not necessary for transposition. The Tc1 transposase, itself along with the terminal 26 bp of the repeat region, are sufficient for activity (Vos et al. 1996).

Although Tc1 was originally found in C. elegans and most of the early research went into the mechanisms of transposition, recent effort has been invested in characterizing the phylogenetic extent of this transposable element. Tc1 has been found in fungi (Langin et al. 1995), nematodes (Emmons et al. 1983), arthropods (Jacobson et al. 1986) and vertebrates (Radice et al. 1994). Tc 1 also shares its DDE motif with bacterial IS transposases and retroviruses (Fayet et al. 1990), leading to the theory that

Tc 1 is a very ancient and conserved transposon. The discovery of the mariner transposable element in Drosophila mauritiana led to the realization that Tc 1 and mariner constituted a 'family' (Jacobson et al. 1986). Transposable elements belonging to this family subsequently have been discovered in species representing several lineages, several with Tc1 elements that are still active. One lineage shown to lack such a Tc1-like element is the ray-finned fish clade. Almost all remnants of elements found have been shown to have stop codons within their open reading frame. There is evidence, however, for activity in a few species. In zebrafish, active transposition of Tc1-like elements have been shown to occur, although no Tc1-like element with a complete open reading frame has been found to date (Lam et al. 1996). There have also been transcribed Tc1-like transposons found in two groups of salmonid fish, though these Tc-1 like transposable elements have premature stop codons and have not been shown to be translated into functional transposases (Krasnov et al. 2005).

### 5.2 Transposable Elements in Hox Clusters

Transposable elements were originally thought to be excluded from gnathostome Hox gene clusters due to their ability to cause genomic rearrangements, alter intergenic distances and ablate or introduce cis-regulatory elements (Fried et al. 2004). But, recent work has shown there are indeed TEs within the Hox gene clusters (Di poï et al. 2009). We have systematically searched Hox gene clusters of the following gnathostomes for transposable elements: horn shark, human, coelacanth (Latimeria menadoensis), bichir, zebrafish, medaka, Nile tilapia, spotted-green pufferfish, Western clawed frog, and chicken (Figure 24).

From this analysis we discovered that TEs do reside in and around Hox gene clusters of gnathostomes although the type of TEs differs between lineages. Human and shark and for the most part coelacanth Hox clusters contain class I transposons whereas the ray-finned fishes including bichir Hox clusters contain mostly class II transposons that increase in frequency in the teleost division. This shows a distinction between rayfinned fishes and cartilaginous/lobe-finned fishes. This distinction becomes relevant in terms of how these two types of transposons transpose. Class II transposons "cut and paste" to adjacent regions of DNA with sloppy precision taking with and leaving behind DNA sequences whereas class I transposons "copy and paste" resulting only in insertions. Because of this class I transposons have a greater ability to alter intergenic distances.

One specific class II transposon was of interest due to finding two open reading frames in and immediately flanking the bichir Hox cluster. The Tc1/mariner family of DNA transposons is found throughout animal phylogeny from C. elegans to Drosophila
to frog (Emmons et al. 1983; Harris et al. 1988; Lam et al. 1996). It is also found among many teleosts fishes (Radice et al. 1994). However a Tc1 with an intact open reading frame has never been found in a vertebrate. We have found two Tc1 transposons in and around the bichir Hox clusters. The first is found flanking bichir's HoxA cluster just downstream of HoxA1. The second is found within the 16,254 base pair intron of HoxD3. This is the largest intron found to date for a canonical Hox gene. A typical Hox gene intron is $\sim 25 \mathrm{X}$ smaller (200-700 base pairs). These two Tc1 transposons both contain complete open reading frames that code for amino acid sequences that share homology with known functional Tc 1 transposons including the DDE active motif, glycine rich box, nuclear localization signal and paired DNA binding domains (Figure 25). They also contain the inverted repeats flanking the open reading frames that are necessary for self-excision. Although these two Tc1 transposons both appear functional they do differ from each other significantly and do not appear to be the result of a recent transposition from one location to the other (Figure 25).

Tc1 family members are grouped into different sub-families. Within the teleost division Tc1-like transposons are grouped into $\mathrm{A}, \mathrm{B}$ and C subfamilies with A and B being more closely related (Ivics et al. 1996). Other, more ancient, Tc1-like sequences from a diverse array of organisms (C. elegans, frog, fungus, etc.) group outside of the recent teleosts expansions and appear more closely related to one another than to any of the 3 teleost subfamilies (Figure 26). The Tc1-like transposon flanking bichir's HoxA cluster (bichir HoxA Tzf) groups with subfamily C, which is mainly composed of zebrafish Tc1-like sequences. The Tc1-like transposon found with bichir's HoxD3 intron (bichir HoxD Tc1) groups with the seemingly more ancient clade. The HoxD Tc1 also
differs in the spacing of its catalytic domain. Tc1 family members normally have a DD34E catalytic domain with 34 amino acids between the second aspartate and the glutamate (Shao and Tu 2001). Other variations have also been found for related transposons such as the DD35E motif shared by the bacterial insertion sequence (IS) and human immuno-deficiency virus (HIV, Plasterk et al. 1999). DD37E transposons have also been found (Shao and Tu 2001). The HoxD Tc1 has 36 amino acids between the second aspartate and glutamate (Figure 25). The only other transposon with the DD36E configuration is the Tc1-like transposon Maya2 found in the African clawed frog (Sinzelle et al. 2005). HoxD Tc1 also shares the short inverted repeats with Maya2 and the original Tc 1 found in C. elegans.

Tc1-like sequences are found in and around Hox clusters of the teleost fishes as well (Figure 27). They maintain the pattern seen in bichir where subfamily C members flank the Hox gene clusters and the seemingly more ancient Tc 1 transposons are located within Hox gene clusters. In zebrafish, 4 subfamily C members are found within 100 kilobases upstream of the HoxAa cluster and 2 are found within 50 kilobases downstream and all contain inverted repeats. This is significant because Tc1-like transposons normally transpose to an area within 100 kilobases from where they are excised (Fischer et al. 2001; Carlson et al. 2003). Also, intact inverted repeats within 12.5 kilobases of each other are all that are necessary for the Tc 1 transposon protein to excise and transpose DNA (Fischer et al. 1999). At first glance this may not seem an issue in zebrafish since no intact Tc 1 has been found that is capable of producing a full-length protein. However, as previously mentioned it has been shown that zebrafish has Tc1 transposon activity (Lam et al. 1996). Whether there is another transposon capable of
utilizing the Tc 1 inverted repeats is unknown, but it is also possible that the intact Tc 1 has yet to be annotated due to the incompleteness of the genome sequence. There is also a subfamily C member flanking the anterior end of the zebrafish HoxDa cluster. Within the Hox clusters are only members that group with the seemingly more ancient clade. There is one between zebrafish's HoxA11a and A9a, HoxB13a and B10a, HoxB2a and B1a, HoxC3a and C1a and medaka's Eve1 and HoxD13a and HoxD13a and D9a. It appears that at the time the more ancient Tc1-like transposon entered the teleost fishes' genomes the Hox gene clusters were more amenable to invasion but during the more recent expansion of the teleosts-specific Tc1-like transposon expansion they have been excluded.

### 5.3 Testing Tc1 Transposon Activity

Since we found two intact Tc1 transposons in and around bichir's Hox clusters we decided to develop both theoretical and functional tests to determine the ability of these sequences to create a functional tranpsosase as well as their ability to alter the structure of the Hox clusters in which they reside.

First, we determined if there was a difference in the likelihood that a Tc 1 would jump into Hox clusters of different species since we had previously determined that there is a difference in the types of transposons that invade ray-finned fishes Hox clusters and lobe-finned and cartilaginous fishes Hox clusters. In order to perform this analysis we implemented the ProTIS program developed by Liu et al. (2005). In this program 4 types of Tc1-like insertion sites are given weights based on empirical frequencies of insertion. The four categories are $13,5,4$, and 1 where 13 is 13 times more likely to incur an insertion than 1. This script was run in Perl on the HoxA clusters of horn shark, human, mouse, coelacanth, bichir, zebrafish (Aa and Ab) and Japanese pufferfish (Takifugu rubripes; Figure 28, 29). From this analysis it is apparent that it is twice as likely that a Tc1-like transposon will jump into an intergenic or intronic region rather than an exonic region. It is also apparent that the species that actually have Tc1-like sequences in an around their Hox clusters are twice as likely to have an additional insertion of a Tc1-like transposon within their Hox clusters. Bichir and zebrafish have a relative average weight of 0.40 for their non-coding regions whereas mouse and human have a relative average weight of 0.15 . This discrepancy disappears outside of the HoxA cluster where bichir and zebrafish have a relative average weight of 0.25 and mouse and human 0.22 . From this data we can conclude that the reason ray-finned fishes have invasions of class II
transposon where lobed-finned fishes do not is partly due to their respective Hox cluster sequence preference. It appears that mouse and human actively maintain an environment that is not conducive to Tc 1 transposon insertion (average weight of 0.15 inside cluster to 0.22 outside cluster) and bichir and zebrafish are maintaining an environment that is conducive to Tc 1 transposon insertion (average weight of 0.40 inside cluster to 0.25 outside cluster).

Second, we determined what would be the results of a Tc1-like transposon jumping in and out of the bichir HoxA cluster. For this analysis a program was written in Perl and JAVA with the help of lab members (Yu-Kang Cheng, Richard David-Rus and Robert Lynch). In this implementation the program takes into consideration the location of the ORF of the transposon, the inverted repeats, Hox coding regions and pattern of insertion sites (obtained by previously mentioned script). Variables for the program include rate of fixation in generations, nucleotide mutation rate, population size, number of fixation events and number of runs to execute. For our analysis we started out with the following parameters:

Fixation Rate: 1
Mutation Rate: 0.000624855
Population Size: 500
Fixation Events: 25
Current number of runs used: 1000
In summary the algorithm would jump the transposon and check for sequence differences. If the mutation was in an exon then it was considered inviable and a new mutation was performed. If the mutation hit in a PFC it was considered inviable with an $80 \%$ rate of survival. If the mutation hit another area of the Hox cluster it was considered $100 \%$ viable and continued. In order to multiply the class II Tc1 transposon must jump
in front of the replication fork thus copying itself. We considered this would happen 1 out of every 1000 jumps. In order for the transposase to transpose it must find two inverted repeats within 12.5 kilobases of each other and in the correct orientation (Fischer et al. 1999). Once these are found the transposase will cut this sequence out and jump it either to the right or left with a $50 \%$ probability. If the transposition is outside of the reference sequence then it will be considered missing and the run will start over. When a transposition occurs there are 8 types of footprints left behind. Which type was considered with the frequency of the empirical weights given by van Luenen et al. (1994). Based on this program there appears to be no preference of any specific intergenic or intronic region in which the Tc 1 transposase integrates (Figure 30). Also, the major changes in Hox gene cluster architecture were due to spacing and not sequence alteration.

Third, we developed an in vitro system to test the ability of the Tc1 HoxA and Tc1 HoxD transposons from bichir to mediate transposition. The first step of this experiment was to construct vectors (Figure 31). Two vectors contain the transposase coding sequence of each bichir Tc1 transposon. Four vectors contain reporter genes with inverted repeats from either transposase sequence. As a backbone pFastBac 1 vector (GenBank accession number: AY598466.1) was used that contains its own polyhedron promoter, SV40 polyadenylation site as well as an F1 origin of replication, gentamicinresistance gene and ampicillin-resistance gene. Before each vector was created we first integrated a multiple cloning site (synthesized by IDT) developed to specifically integrate our genes and promoters between the polyhedron promoter and SV40 polyadenylation site. The multiple cloning site also included the short inverted repeats of the Tc1 HoxD
transposon. To each reporter gene vector all additional sequences were added between the inverted repeats. We spliced in the EF1 $\alpha$ eukaryotic promoter (GenBank accession number: EF203084.1) as well as the EM7 prokaryotic promoter (synthesized by IDT) to vectors 1-4. For vector 1 we added the reporter fusion gene HcRed-Blasticidin (GenBank accession numbers: AY92935.1 and AB364162.1, respectively) and for vector 2 we added Hygromycin-green fluorescent protein (GenBank accession number: AB289768.2) downstream of the two promoter sequences. For vector 3 and 4 we replaced the Tc1 HoxD inverted repeats of vectors 1 and 2, respectively, with Tc1 HoxA inverted repeats. For the expression vectors we added all sequences outside of the inverted repeats specifically downstream. We added a CAG promoter (GenBank accession number: EF59149.1) to both vectors 5 and 6 . To vector 5 we added the Tc1 Hox D transposase coding sequence downstream of the CAG promoter. For vector 6 we replaced the Tc1 HoxD transposase coding sequence with the Tc1 HoxA transposase coding sequence. The total size of each vector was approximately and did not exceed the $7,000-8,000$ base pair range. The vectors are identified as follows: vector 1pFBEEH Red, vector 2-pFBEEHygroGFP, vector 3-pFB5EEHcRed3, vector 4pFB5EEHygroGFP, vector 5-pFBCAGpTc1 and vector 6-pFBCAGpTzf.

Vector design, pFastBac1 vector and reporter gene mother vectors were obtained from Rick Cohen's lab of the W.M. Keck Center for Collaborative Neuroscience at Rutgers, The State University of New Jersey. The multiple cloning site and EM7 promoter were synthesized each as two single stranded DNA molecules by IDT. We annealed the sense and anti-sense strands of these sequences via the following protocol: 1. Dilute DNA oligo crystals in Fermentas 1X PNK Buffer A to a final concentration of
0.001 M. a. Add 20 ul of each strand of the multiple cloning site ( 50 ul each of EM7 promoter) to a 1.5 mL tube, hand shake and quick spin. b. Add 3 inches of water to a 150 mL beaker and bring to a boil, remove from heat and place on desktop. c. Place 1.5 mL tubes in a float and place in water, let come to room temperature ( $\sim 45$ minutes). 2. Phosphorylate double stranded oligos. a. add the following to a 1.5 mL tube ( 20 uL dsDNA oligo, 4 uL 10X PNK Buffer A, 0.4 uL 100 uM dATP, 13.6 uL pure water, 2.0 uL T4 polykinase (Fermentas). b. incubate in 37 C water bath for 20 minutes. 3. Follow protocol for Enzyme reaction cleanup (Qiagen MinElute Kit 28204). Use spectrophotometer to measure concentration of purified DNA. Run $1 \%$ agarose gel to check for proper annealing.

The next step is to amplify the promoters, reporter genes and Tc 1 transposons from their respective vectors. Each of these sequences are amplified from their vectors using primers specific for the sequence with additional sequences that can be cut by restriction enzyme to produce overhangs for insertion into vector. They were amplified with the following primers, annealing temperatures and extension times:

Ef1 $\alpha$ (58 C, 45"), Ef1-alpha-ClaI-F (5’-ATATATCGATGTTTGCCGCCAGAACACAGGTAAGTGC-3') and Ef1-alpha-SphI-R (5'-GTATGGCATGCTTTGGCTTTTAGGGGTAGTTTTCACGACAC-3'); HcRed-Blasticidin (58 C, 45"), HcRed-BSD-5-SalI-F (5'-

CATACGTCGACCCACCATGGTGAGCGGCCTGCTGAAG-3') and HcRed-BSD-3-SbfI-R (5’-GTATGCCTGCAGGTTAGCCCTCCCACACATAACCAGAG-3'); Hygromycin-green fluorescent protein (57 C, 1'), HygroGFP-5-SalI-F (5'-

CATACGTCGACCCACCATGAAAAAGCCTGAACTCACCGCGACG-3') and HygroGFP-3-SbfI-R (5'-GTATGCCTGCAGGTTACTTGTACAGCTCGTCCATGCCGAG-3'); CAG promoter (57 C, 1’), CAGpromoter-5-AscI-F (5’-CATACGGCGCGCCCTAGTTATTAATAGTAATCAATTAC-3') and CAGpromoter-3-NsiI-R (5'-GTATGATGCATTTTGCCAAAATGATGAGACAGCAC-3'); Tc1 HoxD (57

C, 1'), Tc1-5-NsiI-F (5'- CATACATGCATCCACCATGCGGCAGTCGTGTGGGCGAAAATGC-3') and Tc1-3-XmaI-R (5’-GTATGCCCGGGCTAACACGGTGTTTGACCCCCTTTCG-3'); Tc1 HoxA (57 C, 1'), Tzf-5-NsiI-F (5'- CATACATGCATCCACCATGTCAGAGCACAAACCAAGCATG-3') and Tzf-3-XmaI-R (5'- GTATGCCCGGGTCAATACTTTGTCGATGCACCTTTG-3'); Tc1 HoxA 5' inverted repeat (55 C, 30"), 5IVRTzf-5-NotI-F (5'- CATACGCGGCCGCTACAGTGCATCCGGAAAGTATTCA-3') and 5IVRTzf-3-ClaI-R (5’-GTGATATCGATAGCAAAGGCTGTGAATACTTATGTA-3’); Tc1 HoxA 3’ inverted repeat (55 C, 30"), 3IVRTzf-MluI-F (5’-CATACACGCGTGCAAAGGCTTTGAATACTTATGTA-3') and 3IVRTzf-AscI-R (5'-GTATGGGCGCGCCTACAGTGCATCCGGAAAGTATTC-3’).

The PCR reactions were setup with the following volumes: 5.0 uL 10X LA Taq buffer (Takara), $4.0 \mathrm{uL} 25 \mathrm{mg} / \mathrm{mL} \mathrm{MgCl}_{2}, 4.0 \mathrm{uL} 10 \mathrm{mg} / \mathrm{mL} \mathrm{dNTP}, 2.5 \mathrm{uL} 10 \mathrm{pmol} / \mathrm{uL}$ forward primer, $2.5 \mathrm{uL} 10 \mathrm{pmol} / \mathrm{uL}$ reverse primer, $30-60 \mathrm{ng}$ DNA template, 0.5 uL LA Taq (Takara), dilute to 50 uL with pure water. The PCR reactions were ran through PCR in a Eppendorf EP gradient $S$ thermalcycler with the following program: initial denaturation 95 C 2’ 30 cycles of ( 95 C 30 ", variable annealing temperature for 1’, 68 C for variable extension time), final extension 68 C 5’. PCR reactions were purified using Quiagen Gel Extraction Kit 28704 under standard protocol conditions.

The next step is to end digest the amplified sequences. Each sequence is digested with specific restriction endonucleases that will allow them to be ligated to the vector in a particular location. The following enzymes were used to end digest each sequence: Ef1alpha, 10 units ClaI (New England Biolabs, NEB) and 10 units SphI (NEB) with 1X bovine serum albumin and NEB buffer 4; HcRed-Blastocidin, 20 units SalI (Fermentas, F) and 20 units SdaI (F) with Fermentas buffer SdaI; Hygromycin-green fluorescent protein, 20 units SalI (F) and 20 units SdaI (F) with Fermentas buffer SdaI; CAG promoter, 20 units AscI (F) and 20 units NsiI (F) Fermentas buffer R; Tc1 HoxD, 20 units

NsiI (F) and 20 units XmaI (NEB) with Fermentas buffer SdaI; Tc1 HoxA, 20 units NsiI (F) and 20 units XmaI (NEB) with Fermentas buffer SdaI; Tc1 HoxA 5’ inverted repeat, 15 units ClaI (NEB) and 10 units NotI (NEB) with NEB buffer 3; Tc1 HoxA 3' inverted repeat, 20 units AscI (F) and 20 units (F) MluI with Fermentas buffer R. Each reaction consists of 5.0 ug of DNA (except Tc1 HoxA 5' and 3' inverted repeats which uses only 500 ng ) along with the restriction enzymes and buffers diluted to 100 uL with pure water. Reactions are placed in a 37 C for 1 hour. Reactions are then purified using Qiagen Enzyme Reaction Cleanup Kit 28204 under standard protocol.

The next step is to ligate the inserts to the vector. First the vector must be digested to receive the proper insert. For any particular insert digest the vector in the same reaction conditions including the same enzymes. Also set up 2 test digestions (10 $u L$ total) one for each enzyme independently. Dephosphorylate the main digestion by the following protocol: 1. Add 5.0 uL 10X Fast AP buffer (Fermentas), 4.0 uL Fast AP (Fermentas), 43 uL sterile water to a 1.5 mL tube and mix. 2. Add mix directly to digestion reaction after digestion and place in 37 C water bath for $10^{\prime}$. Run gel of uncut, single digestions and main digestion on 1\% agarose gel to check for proper digestion. Second the insert should be ligated to the vector. The insert to vector ratio should be 3:1. The ligation should be setup under the following conditions: 100 ng vector, variable amount of insert, 2 uL 10 X ligase buffer (Fermentas), 1 uL T4 DNA ligase (Fermentas \#EL0014) and dilute to 20 uL with sterile water. Place at room temperature for 1 hour. Vector can be transformed into JM109 competent cells ((Promega) using protocol outlined in chapter 4. Colony PCR and sequence verification are performed using protocol outlined in chapter 4.

After the 6 vectors were created they were transformed into human embryonic kidney cell (HEK) 293 cell lines and grown in ampicillin to test for the presence of vector. The vectors have been transformed into the HEK 293 cell lines in the following combinations: 1. negative control-unrelated vector with no expressed genes 2. pFBEEHygroGFP, pFBCAGpTc1 3. pFB5EEHygroGFP3, pFBCAGpTzf. The HcRedBlastocidin reporter gene has yet to be used because transformation of both transposons simultaneously has yet to be performed.

After initial growth in ampicillin surviving cells were transferred to hygromycin plates and scored for fluorescence. Initial results suggest that the Tc1 HoxD transposase was able to mediate transposition of the reporter gene via its inverted repeats into the nuclear genome of the cell.

### 5.4 Conclusion

Transposable elements are themselves mutations (change in DNA sequence) but they also cause mutation. Because of this and the frequency at which they occur they are considered drivers of genome evolution (review, Biémont and Vieira 2006). The Hox gene cluster was thought to be devoid of such elements due to the abilities of these elements to cause large and small-scale genomic rearrangements that would seemingly disrupt the orderly and clustered nature. Shockingly, TEs were discovered in the Hox gene clusters of several species of gnathostomes. However, the types of TEs found in Hox gene clusters seem to be lineage specific. The ray-finned fishes have undergone an invasion of class II transposons that tend to associate with greater recombination rates as opposed to the lobe-finned and cartilaginous fishes, which have an invasion of class I transposons. One particular class II transposon that is highly associated with areas of high recombination, the Tc 1 transposable element, is found in great frequency in and around the ray-finned fishes' Hox gene clusters. This invasion seems to have been specific to a particular type of Tc1 element and perhaps to a particular period in geological time. Closer inspection of these Tc1 elements in and around the ray-finned fishes Hox gene clusters reveals two intact transposons in the basal living ray-finned fish, bichir. Present knowledge indicates that these are the only known intact Tc1 elements in all of Vertebrata. Experiments show that discrimination between types of transposable elements found in Hox gene clusters of different species/lineages is the result of presence/absence of insertion sites within those clusters. These patterns are likely
maintained through purifying selection. This implies that invasion of the Hox gene clusters coincide with relaxed constraints on purifying selection.

As we have shown previously with the higher rate of turnover of CNSs and the faster rate of mutation in the r3/r5 enhancer of the HoxA2 gene, the ray-finned fishes and to a greater extent the derived teleost clade have what can best be described as an 'evolvable' Hox gene cluster system. This evolvability is apparent at both the molecular level as well as the phenotypic level and can be seen at both the micro as well as macroevolutionary levels. Such a system was likely to arise in a fashion similar to the following scenario.

For the ray-finned fish lineage to develop an "evolvable" genome based on Lynch (2007) it is likely that there was a bottleneck in population size(s) that allowed mutation and drift to act as the major evolutionary forces, which led to more complex genes. Following this bottleneck a population expansion necessarily occurred leading to an increase in heterozygotes, which is synonymous with an increase in standing variation. Either following or co-occurring with the population expansion was an increase in natural selection as the major evolutionary force due both to strong extrinsic factors (i.e. niche filling, resource competition, etc.) and to the increase in the effectiveness in selection due to the large population size. Strong extrinsic factors that coincide with the diversification of ray-finned fish include large continental plate movements exposing $1,000 \mathrm{~s}$ of miles of new shoreline, rise of current coral reef species, sea level changes, etc. (Bellwood and Wainwright 2002). Species diversity associated with these extrinsic factors has been studied in pufferfishes and was shown to be significantly correlated with species number (Alfaro et al. 2007).

## Chapter 6 - Overall Conclusions

### 6.1 Questions Addressed

My thesis has addressed the evolution of the intergenic sequences of Hox gene clusters among jawed-vertebrates. Specifically it has concentrated on the current state of Hox gene cluster intergenic sequences in the ray-finned fishes to obtain a better understanding of how these sequences changed starting at the base of the lineage leading into the duplicated paralogs of the crown group, teleosts. I found that the intergenic sequences of cartilaginous and lobe-finned fishes have maintained a relatively stable set of PFCs as has been reported previously. Chiu et al. (2004) found that the ray-finned fishes, however, have had a considerable turnover of PFCs in the HoxA cluster starting at the base of the lineage and this turnover continues well into the teleost division. My work extends this to the HoxB and HoxD clusters by adding data from the phylogenetically important ray-finned fish, bichir. I found that this rate of turnover appears to be different but steady for each Hox gene cluster paralog. This restructuring of Hox gene cluster intergenic sequences of the ray-finned fishes appears to be mostly the result of relaxed constraints on the conservation of PFCs due to the loss of several deeply conserved PFCs although positive selection for novel PFCs cannot be ruled out as a few novel ray-finned fish specific PFCs were also found.

Though the macro-evolution of the Hox gene clusters in the ray-finned fishes appears to be undergoing mostly relaxed constraints it is unclear what is happening at the micro-evolutionary level to obtain this result. To date and to my knowledge the question of what forces are acting at the micro-evolutionary level within Hox gene cluster intergenic sequences has not been addressed. To address this, at least for one specific

CRE, I performed a population level study. Curiously, the strong evidence of relaxed selective constraints seen at the macro-evolutionary level for this CRE appears to be partially the result of strong positive selection at the micro-evolutionary level.

Directional selection on CREs is just one possible force shaping the architecture of the Hox gene cluster intergenic sequences. To address what other possible genetic mechanisms have helped shape these non-coding sequences I also cataloged all of the mobile elements and remnants of mobile elements. I found that the conserved Hox gene clusters of cartilaginous and lobe-finned fishes contain mostly class I (retro-) transposons, but ray-finned fishes with their high rate of PFC turnover contain mostly class II (DNA) transposons.

### 6.2 Contribution to the Field

My work has provided the field of Hox gene cluster evolution with an important database to begin the cataloging of PFCs and other CNSs. My hope is this database will lead to a large consensus database that can be expanded upon as well as studied in a collaborative effort between evolutionary and developmental biologist. Specifically I have contributed to the knowledge that large turnover of PFCs seen in the ray-finned fishes HoxA cluster that appears to be steady from the stem to the crown is not confined only to the HoxA cluster and that this turnover proceeds at various rates between the Hox gene cluster paralogs. I have also contributed to the recent work done by Di poï et al. $(2009,2010)$ in lizards that shows mobile elements have invaded the Hox gene clusters. My work specifically adds data from the ray-finned fishes, which were not included in these studies. Finally, my population level study was the first such work done at the micro-evolutionary level for any Hox gene locus. I hope that my population level research will spur initiatives into similar studies for other regions of the Hox gene cluster system and open a door to a deeper understanding of its evolution.

### 6.3 Questions Raised

The findings of my work raise several intriguing questions. 1. What are the function of the 21 deeply conserved PFCs not associated with any known transcripts? The discovery of these deeply conserved PFCs with no known function desperately calls for research into their possible roles in the Hox gene clusters. 2. What are the roles of transposable elements in Hox gene cluster evolution? Research into the correlation between transposable element invasions, PFC turnover and morphological variability will help not only further the knowledge of Hox gene clusters but genome evolution in general. 3. What knowledge can be gained by studying the micro-evolution of the Hox gene clusters? Evidence of strong positive selection at the micro-evolutionary level in an enhancer of a gene that appears to be under relaxed constraints at the macro-evolutionary level brings into question what forces are acting at the micro-evolutionary level in other parts of the Hox gene clusters (microRNAs, coding regions, introns, etc...)

### 6.4 Future Research

Further work is necessary to further realize the impact of the findings of my work. Expression vector studies need to be done to obtain at least a rudimentary knowledge of the function of the deeply conserved PFCs with no known function. While these PFCs may be maintained for reasons other than cis-regulation this is the first logical step. Complete sequencing of Hox gene clusters of species in phylogenetically important positions (such as hagfish, lamprey, lungfish, bowfin, gold eye, etc...) needs to be completed to obtain a better picture of the evolution of the vertebrate Hox gene cluster system. This is exemplified in the recent work in lizards (Di poï et al. 2009, 2010). Collaborative efforts between the fields of mobile element evolution and Hox gene cluster evolution could result in the enrichment of both as each provides a unique mechanism for research and could enrich the other.

To follow up on the evidence of strong positive selection between the sister species of killifish there is a need to first establish the effects on expression of these nucleotide differences and then to link this to a phenotypic difference. Functional analyses of the nucleotide differences in the CRE proved difficult. Perhaps this is due to the relatively few differences between the sister species. It is possible that these changes do not alter whether or not a protein will bind but at what strength and length of time it will bind. If the differences are this slight then binding conditions would have to be optimal to maintain pristine protein structure. Freckleton et al. (2009) have designed one type of analysis that may help overcome this problem. In this study genomic DNA is fragmented and ligated into bacteriophages. These bacteriophages will produce amino acid sequences from the genomic DNA inserts and present these molecules on their
capsid. These phages can then be selected for by binding to a specific DNA sequence (cis-regulatory element). The genomic DNA sequence can then be amplified and either bound to a micro array or sequenced. This would allow the possible production of proteins that are normally only produced in developmental stages of species such as $F$. olivaceus and $F$. notatus where lab strains have not been established. After establishing expression differences, phenotypic correlation studies should also be performed to see if there is a correlation between genotype and phenotype in these fish. The possibility of a connection between the selection seen at the HoxA2b enhancer and the ability to flare the opercle in mate defense are intriguing. Bone morphometrics and behavior studies in these two species could help resolve the results of this selective pressure.

### 6.5 Concluding Remarks

Less than 10 years ago was an idea that the gnathostome Hox gene cluster intergenic sequences, specifically the CNSs, were as conserved as the genes themselves. This was challenged by Chiu et al. (2002) where they showed that in the teleosts Hox gene clusters intergenic sequences had lost several PFCs. This was then attributed to the FSGD. This was again challenged by Chiu et al. (2004) where they showed that the actual turnover of PFCs started well before the FSGD in the basal ray-finned fish, bichir. My research has shown that these changes exist across all Hox gene cluster paralogs although with varying rates of turnover. It was also the convention that mobile elements would be absent from the Hox gene clusters due to their ability to disrupt the order and orientation of the genes in the cluster as well as the spacing between them. My research in ray-finned fishes along with work done by the Duboule lab (Di poï et al. 2009, 2010) in lizards has shown that Hox gene clusters do in fact have mobile elements throughout and that the types of mobile elements that have invaded the clusters are lineage specific. Finally, prior to my research, there has never been a population level study of a Hox gene cluster locus. My findings, specifically the evidence of strong positive selection at the population level along with the unique evolution of ray-finned fishes Hox gene clusters, should focus attention not only on the macro-evolution of this unique system but also on its micro-evolution.

## 7. Legends

### 7.1 Table Legends

Table 1-Tests of Selection
A. Interspecies comparisons. Shown are the percent differences found between F. olivaceus, F. notatus and F. catenatus at several loci. Total differences is the number of differences seen for each comparison and the total length of that comparison followed by the percent difference. Coding/in element is the number of differences seen among nucleotides that either change the amino acid coded for or reside within a known response element. Non-coding/out element is the number of differences seen among nucleotides that either do not change the amino acid coded for or reside outside of a known response element. $K a / K s-K b / K i$ is the results of synonymous/non-synonymous or within binding site/outside binding site ratio tests. The highest ratio is 7.78 seen between sister species F. olivaceus and F. notatus for the Hox A2b Enhancer. B. Intraspecies comparisons. Shown are the percent polymorphisms for each category within $F$. olivaceus populations as well as their $\mathrm{Kb} / \mathrm{Ki}$ ratios for the Hox A2a and Hox A2b enhancers as well as a portion of intron 7 of the PGK1 gene. Also shown are the alleles for each locus and their percent in each population. There are no known binding elements within PGK1 intron 7 therefore no $\mathrm{Kb} / \mathrm{Ki}$ ratio was done. Note the relatively high number of polymorphism within PGK1 intron 7. C. Hardy-Weinberg Test. Shown is the Hardy-Weinberg test for assortative mating for the Hox A2b enhancer within the Rockford Beach sub-population of F. olivaceus. Note that the observed deviation from expected frequencies is $61 \%$ probable to happen by chance thus this population is shown to be in Hardy-Weinberg equilibrium.

Table 2-Primers
List of primer names and sequences that were used to amplify regions of interest used in this study. Annealing temperatures are listed to the right. AB/LA stands for annealing temperatures used for Applied Biosystems Taq polymerase and Takara LA Taq polymerase. Phusion refers to annealing temperatures used for Finnzymes Phusion Taq polymerase.

Table 3-Divergence Estimation between Fundulus sp.
Divergence date estimations for the three Fundulus $s p$. involved in this study along with percent difference between two pufferfish species with an estimated divergence date of 5-30 my. Personal communication with Michael Alfaro.

## Table 4-Exclusive PFC Patterns in Hox A and Hox B Clusters

Percentage of PFCs shared exclusively between indicated groups. Each
comparison was also scaled to the number of pair wise comparisons made for that group of comparisons.

### 7.2 Figure Legends

Figure 1-Oral and Pharyngeal Jaws
Teleosts fishes have two sets of jaws. The oral or frontal jaws have mostly lost the ability to grind or smash foodstuffs. The pharyngeal jaws evolved secondarily and mostly grind and smash foodstuffs. Liem's Hypothesis states that this partitioning of work allows the two jaws to evolve independently.

Figure 2-Metazoan Tree

The Metazoan tree shown here has families as the terminal branches and an emphasis on the ray-finned fishes (Actinopterygii). Two insets show alternative phylogenies for the Actinopterygii.

Figure 3-Gnathostome Phylogeny and Hox Gene Cluster Content
This phylogenetic tree is shown to emphasize the gnathostome species that contain 4 Hox gene clusters versus those that contain 8 Hox gene clusters as a result of the FSGD. For each species or group a summary of known Hox genes are shown to the right. Paralogous group Hox genes are aligned vertically with the corresponding group number at the top $(\mathrm{E}=$ even skipped homologs $)$. For every group/species their Hox clusters are labeled according to accepted nomenclature. A colored pie piece indicates presence of intact Hox gene, a gray pie piece indicates a pseudogene, a white pie piece and/or no pie indicates absence of Hox gene/s. For killifish and striped bass known and verified Hox gene content is listed. See figure 2 for scientific names corresponding to common names.

## Figure 4-Bichir Anatomy

The bichir is a unique organism that shares traits with many different orders as well as some unique features.

Figure 5-Bichir Hox Gene Trees
Bootstrap or Bayesian posterior probability support is indicated for each branch. Protein trees clockwise from top left, neighbor joining, maximum parsimony and Bayesian. Nucleotide trees from top left, neighbor joining, maximum parsimony, maximum likelihood and Bayesian.

Figure 6-Bichir Hox Gene Content

Hox gene cluster size is roughly to scale. Hox genes are indicated by boxes with their paralog identity indicated by number. Hox genes in blue were previously annotated and those in yellow were annotated during this work. Curved lines on either side of a gene indicate incomplete sequencing of that gene. Unconnected Hox genes indicate unsequenced intergenic regions. Hox D3 has a 16,255 bp intron indicated by a line connecting the two coding regions.

## Figure 7-Bichir Hox Gene Trees

Bootstrap or Bayesian posterior probability support is indicated for each branch. Protein trees clockwise from top left, neighbor joining, maximum parsimony and Bayesian. Nucleotide trees from top left, neighbor joining, maximum parsimony, maximum likelihood and Bayesian.

Figure 8-Phylogentic Footprint Clusters Naming Scheme
Proposed nomenclature for naming phylogenetic footprint clusters. The name consists of a 3-letter abbreviation for the species consisting of the first letter of the genus and first two letters of the species. A capital letter representing the Hox cluster, two subscript capital letters represent the position along the cluster, an identification number specific for each PFC, and 1-6 lower case subscript letters representing the section content of that species.

Figure 9-Deeply Conserved PFCs
A map of the locations of deeply conserved phylogenetic footprint clusters on reconstructed gnathostome ancestor HoxA, B, C, and D clusters. Black boxes denote Hox genes; open boxes denote Hox genes that are found in only 1 extant gnathostome to date. Red lines represent PFCs that are found within untranslated regions (UTRs) of Hox
gene mRNAs. Blue lines represent PFCs that are found within 500 nucleotides of a Hox gene proper region but have not been identified as UTRs. Green lines represent PFCs that have high or identical sequence identity to sequences in the EST library. Yellow lines represent PFCs that are not found in any databases or published papers. Black lines are sequences with known function and labeled accordingly. 'Deeply Conserved' refers to PFCs conserved between at least 2 of the following groups, 1) horn shark, 2) $\geq 2$ lobefinned fish, 3 ) bichir, 4$) \geq 2$ teleost fish. The C8 early enhancer does not qualify as deeply conserved but has been found by the PFC method with the 5 ' end conserved in lobefinned fish and the 3' end conserved in teleost fish. Information on blast results and PFC number for each PFC represented in this figure is available in supplementary table 1. Numbers in the figure refer to the following references: 1. Larochelle et al. 1999. 2. Nonchev et al. 1996. 3. Berezikov et al. 2005. 4. Shashikant et al. 1995. 5. Morrison et al. 1996. 6. Yekta et al. 2004. 7. Doersken et al. 1996.

Figure 10-PFC Retention Mosaicism
Select species showing the pattern of PFC retention with species containing 4 Hox gene clusters (red) or 8 Hox gene clusters (blue) for the Hox A (left) and Hox B (right) gene clusters. As you can see from the pie diagrams human retains mostly PFCs that are shared with other 4 Hox gene cluster species whereas bichir has a greater turnover sharing more PFCs with 8 Hox gene cluster species. The same pattern can be seen for zebrafish ' $a$ ' paralogs indicating further turnover within the teleosts. Also the rate of turnover is greater for the Hox B gene cluster than for the Hox A gene cluster.

Figure 11-Hox A Gene Cluster Retention

A simplified phylogenetic tree of the Hox A clusters. Solid circles denote acquisition of a new PFC in that lineage. Open circles denote loss of PFCs in that lineage. Color of open circles denote where the lost PFCs were originally acquired. Numbers indicate the amount of PFCs gained or lost. I only used PFCs that met the criteria of being conserved between at least 3 species. Notice that there is no apparent gain of any PFCs at the base of the teleost but there is evidence of 7 new PFCs gained before the divergence of bichir at the base of the ray-finned fish clade. This puts acquisition of new elements prior to FSGD at a ratio of 7:2. If the two gains in the teleost Aa and Ab clusters are considered to be gained prior to the divergence of the paralogous clusters and then lost in each of the paralogs; the acquisition of new elements in the rayfinned fish clade is in favor of pre-FSGD with a ratio of 9:0.

Figure 12-Intergenic Distances with Mammalian Proxies
Comparison of Hox cluster orthologous intergenic region distances between teleost fish with proxies from mammals. The random bar consists of an average distance for comparisons of 10 random intergenic regions, distances greater than distances between random sequences are considered by chance and therefore are not considered informative. No HoxC cluster is currently available for opossum, Monodelphis domestica. Notice the greater distances for teleost fish opposed to the mammalian proxies. This is either due to an overall increased mutation rate and/or incorrect divergence dates for teleost fishes resulting in incorrect proxies.

Figure 13-Hox A2 Gene Expression Pattern

In mammals and birds the Hox A2 gene is found to be expressed in rhombomeres 2-7 of the developing hindbrain as well as in neural crest cells derived from rhombomere 4. Hox A2 has the most posterior expression of any Hox gene.

Figure 14-Hox A2 Paralogs Expression Patterns
Expression patterns of the Hox A2a and Hox A2b paralogs in various teleost fishes. The Hox A2a paralog maintains a mores ancestral expression pattern in the Acanthomorpha but has been independently pseudogenized in both zebrafish and pufferfish. The Hox A2b paralog has a more varied expression with pseudogenization in medaka.

Figure 15-Hox A2 Gene Regulation Schematic
Hox A2 gene expression is controlled by 3 cis-regulatory elements. The r3/r5 enahncer lies 1-3 kb upstream of the translational start site and controls expression in rhombomeres 3 and 5 as well as neural crest cells derived from rhombomere 4. The r4 enhancer lies within the intron and controls expression in rhombomere 4. The r2 enhancer lies within the $3^{\prime}$ portion of exon 2 and controls expression in rhombomere 2. Figure 16-In-situ Hybridization of Fundulus heteroclitus Embryos

Hox A2 gene expression in Fundulus $s p$. was discovered using RNA in-situ hybridization assays. Hox A2a expression is on the left in each developmental stage and and Hox A2b expression is on the right. Dorsal views are on top and lateral views are on bottom. An arrow indicates the otic vesicle, which lays overlapping rhombomeres 4 and 5. An arrowhead indicates pharyngeal arch 2. EGR2, a krox-20 ortholog, stains rhombomeres 3 and 5 and is used to show location of rhombomeres in early embryos. M and N are 2-4 somite stage embryos with EGR2 staining in red and Hox A2 staining in
blue/purple. A-D are 8 -somite stage embryos. E-H are 14-somite stage embryos. I-L are 21-somite stage embryos.

## Figure 17-Fundulus sp. Hox Gene Trees

Nucleotide trees are shown. Bootstrap or Bayesian posterior probability support is indicated for each branch from top left, neighbor joining, maximum parsimony, maximum likelihood and Bayesian. The branch that supports paralog status for Fundulus $s p$. is circled. Fundulus sp. are circled.

Figure 18-Charater State Trees
This character state tree of the Hox A2 r3/r5 enhancer from NC2 to RE3 including RE4 and 5' portion of NC3 shows the number of nucleotide changes on each branch and branch lengths are proportional to number of changes. 'a' following common names refers to the Hox A2a enhancer and 'b' to the Hox A2b enhancer. Total number of changes on each branch is inside squares and number of single-step changes is in diamonds. The Hox A2b enhancers show significantly more changes among teleosts and those changes are continuously steady moving from the stem to terminal branches. The Hox A2a enhancers share the majority of their changes and have very few terminal branch changes. Killifish Hox A2b enhancers have the most number of changes as well as twice as many single-step changes as any teleost Hox A2 enhancer. The same pattern is seen for the Hox A 2 gene region.

Figure 19-Ka/Ks Ratio and Synonymous Percent Difference
This figure shows the $\mathrm{Ka} / \mathrm{Ks}$ ratios and synonymous percent differences between F. olivaceus and F. notatus for the Hox A2a an Hox A2b r3/r5 enhancers and surrounding genes.

Figure 20-RE4/NC2 Hox A2b r3/r5 Enhancer Variable Region
All 4 fixed differences and one polymorphism are located within 38 bps of each other in the RE4/NC2 region of the $\mathrm{r} 3 / \mathrm{r} 5$ enhancer of the Hox A2b gene in the Fundulus sp. F. notatus has 4 differences that result in slight alteration of RUSH1- $\alpha$ site strength and loss of the Mafb binding site with gain of an HMG-1 binding site. F. olivaceus has 4 differences that result in slight alteration of RUSH1- $\alpha$ site strength and loss relocation of an Ubx site with. F. olivaceus allele \#5 has an additional polymorphism that results in the loss of the RUSH1- $\alpha$ site and does not appear in the population in the homozygous state.

## Figure 21-Master TFBS Retention

Shown are the TFBS for krox-20, AP-2 and Mafb. These three transcription factors control 3 major aspects of Hox A2 gene expression in the developing hindbrain. Response elements 2-5 are also indicated. Triangles indicate AP-2 binding sites and circles indicate Mafb binding sites. Dark color represents TFBS located in the same orientation as transcription and reddish color represents TFBS located in the opposite orientation of transcription.

Figure 22-Fundulus sp. Tree
3 major groups of Fundulus are shown along with their possible phylogenetic relationships. Pictures of $F$. olivaceus and $F$. notatus are also shown showing the presence or absence of spots above the lateral line. Approximate divergence dates are given.

Figure 23-Tc1 DNA Transposon Schematic

Schematic of the original Tc1 DNA transposon found in C. elegans. D refers to the aspartate amino acid and E to the glutamate amino acid. The Tc1 transposon bind directly to its own inverted repeat sequences as a dimer bringing the two inverted repeats within close proximity.

Figure 24-Hox Gene Cluster Transposon Content
The Hox gene clusters of 9 species are represented. The top line represents the Hox gene cluster of amphioxus which contains the most Hox genes of any cluster. The next four lines represent the $\operatorname{Hox} \mathrm{A}, \mathrm{B}, \mathrm{C}$ and D gene clusters (respectively) of bichir (light blue), horn shark (red), coelacanth (green) and human (pink). The last four lines represent the Hox $\mathrm{Aa} / \mathrm{Ab}, \mathrm{Ba} / \mathrm{Bb}, \mathrm{Ca} / \mathrm{Cb}$ and $\mathrm{Da} / \mathrm{Db}$ gene clusters (respectively) of pufferfish (light blue), Nile tilapia (purple), medaka (green) and zebrafish (fuchsia). The bars indicated the percentage of the intergenic region that is composed of transposons. The bars are color coded to each species and designate 3 types of transposons; Class II transposons (solid bar), Class I LTR transposons (striped bar) and Class I non-LTR transposons (open bar).

Figure 25-Tc 1-like Transposons in Bichir
Amino acid alignment of the artificially constructed and functional sleeping beauty Tc 1 -like transposon, the consensus reconstructed ancestral salmonid Tc1-like transposon, the intact Tc1-like transposon found flanking bichir's Hox A gene cluster and the intact Tc1-like transposon found within the intron of bichir's Hox D3 gene. 4 functional domains are highlighted; two DNA binding domains (Paired-like domain with Leucine Zipper and Homeo-like Domain), nuclear localization signal, glycine-rich box and DDE active motif. The DDE active motif is well conserved between the functional
sleeping beauty transposon and the two bichir Tc1-like transposons although the Hox D Tc1-like transposon has a hereto for unseen DD36E motif. Although the DNA binding domains do not appear to be conserved, the two Tc1-like transposons in bichir still are reported as having a Tc1-like DNA binding domain by NCBI protein-blast domain searching algorithm.

Figure 26-Tc 1-like Transposon Tree
The Tc1-like transposons found in teleosts are separated into 3 main groups labeled A, B and C. The Tc1-like sequences found during this study are circled in red. Figure 27-Tc 1-like Tranposons in Hox Gene Clusters

All Tc1-like transposons found within the Hox gene clusters either group or have a greater similarity with Tc1-like sequences that group outside the three main teleosts groups. All Tc1-like transposons found flanking the Hox gene clusters group within teleosts group C. Tc1-like transposons that still maintain an identifiable inverted repeat are identified with a star.

Figure 28-Hox A Gene Cluster Tc1 Insertion Sites
Shown is the relative amount of Tc1 insertion sites found in intergenic regions of 8 Hox A gene clusters. The amount is based on the number and weight of insertion sites. Figure 29-Hox A Gene Cluster Tc1 Insertion Sites supplement

A-D. Shown is the relative amount of Tc 1 insertion sites found in intergenic regions of 8 Hox A gene clusters. These figures show the relative amount divided into the 4 weight categories. E and F. Shown is the relative amount of Tc1 insertion sites found in intron and exon regions, respectively, of 8 Hox A gene clusters.

Figure 30-Simulation Results of Tc1-like Transposition in Bichir Hox A Gene Cluster

Shown is the number of base pair alterations after 25 simulated fixations or "jumps" of the Tc1-like transposon found flanking bichir's Hox A gene cluster. There appears to be no discernable pattern or preference for location of base pair alteration.

Figure 31-Schematic of Tc1-like Activity Confirmation Vectors
All vectors used a pFastBac1 backbone with an integrated multiple cloning site designed by Rick Cohen. $\mathrm{P}_{\mathrm{PH}}$ indicates the polyhedron promoter; SV40 polyA indicates location of the poly A signal. 1-6 shows the different multiple cloning site regions used to make the 6 types of vectors used in this study.

## 8. Tables and Figures <br> 8.1 Tables

Table 1.


3 PGK1 intron7
a. F. olivaceus Polymorphism

Population
Rockford Beach
Gasconade River
Gasconade River
Total
b. F. olivaceus Alleles

Allele
\#1
\#2 AA55GT, G62T, C68T, A72C, A87G,
Total Polymorphism
$6 / 333$
$8 / 333$
$6 / 333$
8/333 (2.4\%)

CCGA264TCTT
\#3 C179T
\#4 C235T
Chromosome count

| Little Piney/Gasconade River | Rockford Beach |
| :---: | :---: |
| $13 \%$ | $65 \%$ |
| $86 \%$ | $22 \%$ |
|  |  |
| $0 \%$ | $8 \%$ |
| $0 \%$ | $4 \%$ |
| 19 | 56 |

Table 2.

|  |  | Annealing |
| :---: | :---: | :---: |
| Primer Name | Primer Sequence | AB/LA-Phusion |
| A9b forward | 5' CTT TGG AGA CGC ACA CWC C 3' | 57 C |
| A9b reverse | 5' TTC TTC ATC TTC ATY CTG CGG 3' |  |
| A2b forward | 5' CCT GAC ATC TCT CGC TAA CC 3' | $59 \mathrm{C}-60 \mathrm{C}$ |
| A2b reverse | 5' AGA GGT CAG ACG CTG CTG C 3' |  |
| A3a forward | 5'ART ACA ARA AGG ATC AGA AAG G 3' | 57 C |
| A3a reverse | 5' TTR CCC ATT GTG ATT GCT CC 3' |  |
| A2a forward | 5' GAA TTC GAR CGA GAG AGC G 3' | $56 \mathrm{C}-60 \mathrm{C}$ |
| A2a reverse | $5^{\prime}$ ACR GGT CCG TTK GAG ATG G 3' |  |
| B2a forward | 5' CAT TTC AAA CTT CAT CAA TCA AGG 3' | 58 C |
| B2a reverse | 5' CTC YTT CAT CCA KGG RAA CTC 3' |  |
| A3a-A2a int 1 forward | 5' AAT GTG AAG CCG TCC GTC C 3' |  |
| A3a-A2a int 1 reverse | 5' CAG TAG CTC TCA TAT TTA GGC 3' |  |
| A3a-A2a int 2 forward | 5' CCA TGC CAC TAG ATT GCA AGC 3' |  |
| A3a-A2a int 2 reverse | 5' ACG TCT ACG TCT CCAAAT CCG 3' |  |
| A3a-A2a int 3 forward | 5' AAC AAA TGC TGG ATA TAG ATG GC 3' |  |
| A3a-A2a int 3 reverse | 5' CAC GTA ACA GTA ACA TGA AGG C 3' |  |
| A2b enhancer forward | 5' AAC ACC CAC TCA CCT CAG C 3' | $56 \mathrm{C}-60 \mathrm{C}$ |
| A2b enhancer reverse | 5' GTG TGA TCA ATC TTT CHM GCC $3^{\prime}$ |  |
| A2a enhancer forward | 5' CAAAGA TAA GTG TGC AGA ACG C 3' | $56 \mathrm{C}-67 \mathrm{C}$ |
| A2a enhancer reverse | 5' TGG CTT GTT GAG TAA GAA GAG G 3' |  |
| PGK1 intron 7 forward | 5' GCR AAG GTG AAA GAT AAG ATY CAG C $3^{\prime}$ | N/A-62 C |
| PGK1 intron 7 reverse | 5' TCT CSG CTT TGG CCA TCA GG 3' |  |
| PGK1 intron 7 short forward | 5' CTG TTT CCA CCA CGA TCT CC 3' | N/A-60 C |
| PGK1 intron 7 short reverse | 5' TCAAAA GAT TGC AAC CAA CG 3' |  |
| A2a exon 2 forward | 5' TGA CCG AGA GGC AGG TCA AGG 3' | 56 C |
| A2a exon 2 reverse | 5' AGG GCA GAG GGG CTG TCA CC 3' |  |
| EGR2 forward | 5' CCA GAC CTT YAC CTA YAT GGG 3' | 59 C |
| EGR2 reverse | 5' TGT GTC TCT TYC TCT CRT CGC 3' |  |

Table 3.

A3a locus (exon 2)
F.olivaceus/F. notatus
F. catenatus/F. olivaceus
F. catenatus/F. notatus
T. nigroviridis/T. rubripes

## A2a locus (exon 1)

F.olivaceus/F. notatus
F. catenatus/F. olivaceus
F. catenatus/F. notatus
T. nigroviridis/T. rubripes

A9b locus (exon1,2 intron)
F.olivaceus/F. notatus
F. catenatus/F. olivaceus
F. catenatus/F. notatus
T. nigroviridis/T. rubripes

## A2b locus (exon1)

F.olivaceus/F. notatus
F. catenatus/F. olivaceus
F. catenatus/F. notatus
T. nigroviridis/T. rubripes

B2a locus (exon1)
F.olivaceus/F. notatus
F. catenatus/F. olivaceus
F. catenatus/F. notatus
T. nigroviridis/T. rubripes

A2a Enhancer
F.olivaceus/F. notatus
F. catenatus/F. olivaceus
F. catenatus/F. notatus
T. nigroviridis/T. rubripes

A2b Enhancer
F.olivaceus/F. notatus
F. catenatus/F. olivaceus
F. catenatus/F. notatus
T. nigroviridis/T. rubripes TOTAL
F.olivaceus/F. notatus
F. catenatus/F. olivaceus
F. catenatus/F. notatus
T. nigroviridis/T. rubripes

| non-coding/out element | 5 | 30 |
| :---: | :---: | :---: |
| 1/151 (0.66\%) | 0.27 | 1.61 |
| 9/151 (5.96\%) | 2.42 | 14.5 |
| 10/151 (6.62\%) | 2.69 | 16.1 |
| 18/146 (12.3\%) | 2.46/my | 0.41/my |
| 1/111 (0.90\%) | 0.45 | 2.73 |
| 1/111 (0.90\%) | 0.45 | 2.73 |
| 2/111(1.80\%) | 0.91 | 5.45 |
| 12/121 (9.9\%) | 1.98/my | 0.33/my |
| 12/407 (2.95\%) | 1.27 | 7.76 |
| 26/407 (6.39\%) | 2.75 | 16.8 |
| 22/407 (5.40\%) | 2.33 | 14.2 |
| 11/95 (11.6\%) | 2.32/my | 0.38/my |
| 2/76 (2.63\%) | 2.37 | 13.8 |
| 0/76 (0.00\%) | 0 | 0 |
| 2/76 (2.63\%) | 2.37 | 13.8 |
| 4/72 (5.56\%) | 1.11/my | 0.19/my |
| 0/71 (0.00\%) | 0 | 0 |
| 1/71 (1.41\%) | 0.56 | 3.36 |
| 1/71 (1.41\%) | 0.56 | 3.36 |
| 20/159 (12.6\%) | 2.52/my | 0.42/my |
| 8/397 (2.02\%) | 0.82 | 4.93 |
| 16/397 (4.03\%) | 1.64 | 9.83 |
| 16/397 (4.03\%) | 1.64 | 9.83 |
| 46/374 (12.3\%) | 2.46/my | 0.41/my |
| 1/210 (0.48\%) | 0.23 | 1.41 |
| 10/210 (4.76\%) | 2.31 | 14 |
| 11/210 (5.24\%) | 2.54 | 15.4 |
| 44/428 (10.3\%) | 2.06/my | 0.34/my |
| 25/1423 (1.76\%) | 0.79 | 4.76 |
| 63/1423 (4.43\%) | 2 | 12 |
| 64/1423 (4.50\%) | 2.03 | 12.2 |
| 155/1395 (11.1\%) | 2.22/my | 0.37/my |

Table 4.
Human

| Raw | 4 cluster |  | teleost |  |
| :---: | :---: | :---: | :---: | :---: |
| HoxA | 48 | 46\% | 57 | 54\% |
| HoxB | 30 | 60\% | 20 | 40\% |


| Scaled | 4 cluster |  | teleost |  |
| :---: | :---: | :---: | :---: | :---: |
| HoxA | 16 | 74\% | 5.7 | 26\% |
| HoxB | 15 | 82\% | 3.33 | 18\% |

Bichir
Raw
HoxA
HoxB

| 4 cluster | Teleost | Both |  |  |
| ---: | :---: | :---: | :---: | :---: |
| 17 | $\mathbf{2 0 \%}$ | 48 | $\mathbf{5 6 \%}$ | 21 |
| $\mathbf{2 4} \%$ |  |  |  |  |
| 3 | $\mathbf{8 \%}$ | 22 | $\mathbf{5 9} \%$ | 12 |
| $\mathbf{3 2}$ | $\mathbf{3 2 \%}$ |  |  |  |


| Scaled HoxA HoxB | 4 cluster |  | Teleost |  | Both |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | 4.3 | 40\% | 4.8 | 45\% | 1.6 | 15\% |
|  | 1 | 17\% | 3.67 | 61\% | 1.3 | 22\% |
|  | Scaled HoxA HoxB |  | 4 cluster |  | Teleost |  |
|  |  |  |  | 47\% | 4.8 | 53\% |
|  |  |  | 2 | 21\% | 3.7 | 79\% |


| Zebrafish |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: |
| Raw <br> HoxAa | 4 cluster |  | teleost |  |
|  | 18 | 49\% | 19 | 51\% |
| HoxAb | 12 | 80\% | 3 | 20\% |
| HoxBa | 23 | 43\% | 30 | 57\% |
| HoxBb | 5 | 71\% | 2 | 29\% |


| Scaled | 4 cluster |  | teleost |  |
| :---: | :---: | :---: | :---: | :---: |
| HoxAa | 3.6 | 49\% | 3.8 | 51\% |
| HoxAb | 2.4 | 71\% | 1 | 29\% |
| HoxBa | 5.8 | 28\% | 15 | 72\% |
| HoxBb | 1.3 | 56\% | 1 | 44\% |

Figure 1.


Credit: Zina Deretsky, National Science Foundation (after Rita Mehta, UC Davis)

Figure 2.
Pharyngeal
Ra Mehta, UC Davis)


Figure 3.


Figure 4.

(Nile Bichir ("Polypterus bichir bichir") from Günther, A.C.L.G., 1880. "An introduction to the study of fishes". Today \& Tomorrow's Book Agency, New Delhi. Category:Polypteriformes)

Figure 5.


Figure 6.

$\$ 5$
4


55
9


Figure 7.


Figure 7. (continued)


Figure 7. (continued)


Figure 7. (continued)


Figure 7. (continued)


Figure 8.


Figure 9.


I UT
I 500 bp up/downstream of gene proper region
\| Sequence homology to EST
Unknown

Figure 10.


Figure 11.


Figure 12.


Figure 13.


Figure 14.

pseudo



Figure 15.


Figure 16.


Figure 17.


Figure 17. (continued)


Figure 18.


Figure 19.


Figure 20.
Ubx 80
FOXL1 84
RUSH-1 $\alpha 88$
FOXL1 86
RUSH-1 $\alpha 91$
Mafb 81
Fca AGCCATATAGCCAGTGAAGCTGTCTACTTCCTGGCCACATATGATC
Fol

Mafb 81
FOXL1 84
UbX 80
Fhe
. . . . . . G . . .

|  | Ubx 80 |
| :---: | :---: |
|  | FOXC1 88 |
|  | FOXL1 89 |
| HMG-1 85 | RUSH-1 $\alpha$ 93 |

Fno.

HMG-1 85 Mafb 81
HMG-1 85

| FOXC1 86 |
| :---: |
| RUSH-1 $\alpha_{90}$ |


| HMG-1 85 <br> FOXC1 $_{86}$ <br> RUSH-1 $\alpha_{90}$ |
| :---: |

Fol5......CG.....T.............................. . . T . . . . G. . . . .

Figure 21.
$\Delta A P-2$ sense
$\Delta$ AP-2 anti-sense

- Mafb sense
- Mafb anti-sense


Figure 22.


Figure 23.


Figure 24.


Figure 25.


Figure 26.


Figure 27.


Figure 28.


Figure 29.
HoxA Cluster Te1 IS (Intergerici/'3)


HoxA Cluster Tc1 IS (Intergenic/5)


Figure 29. (continued)


HoxA Cluster Te1 IS (Intergeniel1)


Figure 29. (continued)


HoxA Cluster Te1 IS (exons)


Figure 30.


Figure 31.


## 9. Appendices

Table S1-PFC Database

Each folder contains an "All species" file that contains a list of all PFCs for that particular Hox cluster paralog. For each PFC there is a name in the cell corresponding to the species that contains that particular PFC. For each individual Hox cluster there is a file that contains all PFCs contained within that Hox cluster, the distance to the 3' Hox gene, the length of the PFC in that Hox cluster, and the sequence of the PFC from that Hox cluster. For the Human Hox cluster files and asterisk has been placed next to the name of all PFCs that also correspond to conserved non-coding sequences found between human (Homo sapiens) and pufferfish (Takifugu rubripes) by Lee et al. (2006).

|  | Homana | Frov $A$ | mamath | Humma A | Bichir $A$ | $\underline{\text { L Lramifh } A A}$ | Stimed Basa | Stin |  | Tetraodon A | $\frac{\text { Tratifug } A A}{}$ |  | ${ }_{\text {Mataba }}^{\text {M }}$ | Tratadan ${ }^{\text {a }}$ | Trukita ${ }^{\text {ab }}$ |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | 2／7MM） |  | Lmeam ${ }^{\text {a }}$ | Hatac ${ }^{\text {a }}$ | Peekct |  |  |  |  |  |  |  |  |  |  |
|  | 3． | X ${ }^{\text {xta } a^{3}}$ |  | Hatac ${ }^{\text {a }}$ |  |  | 年mmm |  |  |  |  | ${ }^{+}$ | ， | － |  |
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|  |  |  |  |  | Preay 7 | DreAs，${ }^{\text {c }}$ | 捽䋛 |  |  |  |  |  | ＋ | H－ | H |
|  | s |  |  |  |  |  | 粹 | 隹 |  |  | FuAAM ${ }^{\text {d }}$ |  | H |  |  |
|  |  |  |  | ${ }_{\text {Heana }}^{\text {Hatact }}$ |  | Dreatar |  |  |  | minact ${ }^{\text {c }}$ |  |  |  | ${ }^{\text {H }}$ H ${ }^{\text {H }}$ |  |
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|  | ${ }^{3} 1 \mathrm{Hffanc3l}$ |  |  |  | ${ }^{\text {Anc3lm }}$ | Dreatackem |  |  | 隹 | mitacklom | matack | eabaside |  |  | 多 |
| ${ }^{32}$ | ${ }^{32}$ |  |  |  |  |  |  |  |  |  |  |  | ${ }^{\text {ABa } 32}$ | ${ }_{\text {Tini } \mathrm{Ba}_{3} 32}$ |  |
| ）${ }^{33}$ | ${ }^{\frac{3}{3} 3}$ |  |  |  |  |  |  |  |  |  |  |  | ${ }_{\text {OLARB33 }}$ |  |  |
| ${ }_{35}$ | ${ }^{35}$ | $\mathrm{XITACa}^{35}$ |  | $\mathrm{Ha}_{3} \mathrm{C}_{3} 3$ |  |  |  |  |  |  |  |  |  |  |  |
|  | ${ }^{36}$ |  |  | $\mathrm{Ha4a}_{4} 36$ |  |  | 鲂 |  |  |  |  |  |  |  |  |
|  | ${ }_{\text {cose }}$ |  |  | ${ }_{\text {Heata }}{ }_{\text {Ham }}$ |  | DreAAca3 |  |  |  |  |  |  |  |  | mmom |
| ${ }^{39}$ | ${ }^{39}$ |  | （ | $\mathrm{HaMa}^{\text {a }}$ |  |  |  |  |  |  |  |  |  |  |  |
|  | ${ }^{40}$ |  |  | Нааасат |  |  |  |  |  |  |  |  |  |  | 磁加紬 |
| $\frac{41}{42}$ | ， |  |  | Heacta |  | DreAcsat |  |  |  | minat |  |  |  |  |  |
|  |  |  |  | ${ }^{\text {Heatache }}$ |  |  |  |  |  |  |  |  |  |  |  |
| ${ }_{4}^{4}$ | ${ }_{45}^{4}$ |  |  |  | $\underbrace{\substack{\text { Peata }}}_{\text {Prearat }}$ | DreAAcat |  |  |  |  |  | Drasa |  |  |  |
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| ${ }_{\substack{48 \\ 49}}$ | ${ }^{48} 8$ |  |  | ${ }_{\text {Hatacas }}$ |  |  |  |  |  |  | TruAd ${ }_{\text {a }}$ |  |  |  |  |
| 500 | 50 |  |  |  |  | DreAAcs ${ }^{\text {a }}$ |  |  | Oinatas |  |  |  |  |  |  |
| $\frac{51}{52}$ | 51 |  |  | Heats ${ }^{\text {S }}$ |  |  |  |  |  |  |  |  |  | nixkas |  |
| $\frac{52}{53}$ |  |  |  |  |  |  |  |  |  |  | $\mathrm{T}_{\text {TuAACS }}{ }^{\text {S }}$ |  |  |  |  |
| ${ }_{54}$ | ${ }^{54}$ |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| ¢ | ¢ |  |  |  |  |  | 隹加加m |  |  |  | TuaAcas |  |  |  |  |
| 57 | 57 |  | Lmed ${ }^{5}$ | Hata ${ }^{\text {a }}$ ， | ${ }_{\text {mema }}$ |  |  |  |  |  |  |  |  |  |  |
| ¢ | 5s， |  |  | Heatas |  |  | － |  |  | TiliAcs ${ }^{\text {a }}$ |  | DraABas | $\mathrm{OLaABa}_{\text {a }}$ |  |  |
|  |  | $\mathrm{Xram}_{\text {a }}$ | Lmeatsoment | Hatactalam | Receacobem | DreAAction |  | mid． |  |  |  | Drasemefem |  |  |  |
| $\frac{6}{62}$ | ${ }_{6}^{6} \mathrm{Hffraveb}^{62}$ |  | $\mathrm{Lmme}_{6} 62$ | $\mathrm{Hasan}_{\mathrm{m}} \mathrm{C}^{2}$ | $\mathrm{PeCA}_{4} 62$ |  |  |  |  |  |  |  | ${ }^{\text {masm }}$ |  | ${ }^{\text {man }}$ |
| ${ }^{63}$ | ${ }^{63}$ |  |  | $\mathrm{Hsatan}_{4}$ | Seat，63 |  |  |  |  |  |  |  |  |  |  |
| ${ }_{6}^{64}$ |  |  |  |  |  | DreAMuche |  |  |  |  | TruA $\mathrm{A}_{6} 6$ Sm |  |  |  |  |
| 6 | ${ }^{66}$ |  |  |  | P．eatrob |  | 災加紬 |  |  | \％intiose |  |  | ${ }^{\text {anaimb }}$ |  |  |
| 6 | 6. |  |  |  | Preativ |  |  |  |  | iniab |  |  |  |  |  |
| ${ }_{6}^{68}$ | ${ }_{6}^{68}$ |  | LmeA $\mathrm{A}_{6}$ 9 |  | Peatus |  |  |  |  |  |  |  | $\mathrm{OLaABG}^{\text {a }}$ |  |  |
| 70 | 70 |  |  |  |  | DreAmim ${ }^{\text {a }}$ |  |  |  |  |  |  |  |  |  |
| ${ }^{\text {PRCC }}$ | Homblark | Fros A | CotacambA | $\frac{\text { Human }}{}$ | Bicitir | 2etrafib AA | smond Bus． | 俍 | Mcdaka AA | Tetrododo $A$ |  | Zebraifid AB | Mcadat AB | Ceraodon AB | Ralifug AB |
| ${ }^{12}$ | ${ }^{2}$ |  |  | Hatal ${ }^{\text {a }}$ |  |  |  | ${ }^{\text {a }}$ |  |  |  |  |  |  |  |
| $\frac{13}{74}$ |  |  |  | HaAar ${ }^{\text {P }}$ | Pseare7 |  | 切加m |  |  |  |  | Dreabur |  |  |  |
| ${ }_{7}{ }^{7}$ | ${ }^{15}$ Hfitav 75 | $\mathrm{xran}^{\text {a }} 75$ |  |  | PbeA． 75 |  |  |  |  | ${ }_{\text {minta }}$ |  |  |  |  | ${ }_{\text {max }}$ |
| ${ }_{77}$ | ${ }_{7} 7$ | $\mathrm{XITAAEP7}^{\text {l }}$ |  | HaAarc7 |  |  |  |  |  |  |  |  |  |  |  |
| ${ }^{78}$ |  | ${ }^{\text {x，}}$ | Limenemp | ${ }_{\text {Hatem }}$ | Peater 7 ， |  |  |  |  |  |  |  |  |  |  |
| $\stackrel{79}{80}$ |  |  |  | Hatame ${ }_{\text {\％}}$ |  | DreAAm9 |  |  |  |  | TruA Acramenter | Drasempme |  |  |  |
| $\frac{81}{82}$ |  |  |  |  |  | Drean ${ }^{\text {S2 }}$ |  |  |  | min ${ }^{2}$ |  | Drasms |  |  |  |
| ${ }_{8} 8$ | ${ }_{8}$ |  |  |  | Pseam ${ }^{83}$ |  |  | $\mathrm{Oinitanc}_{83}$ |  | －matar |  |  |  |  |  |
| \％${ }_{8}^{85}$ | ${ }_{\substack{84 \\ 88 \\ 85}}$ |  | Lmeats 8 | Hatas | Preatus 4 |  |  |  |  | riniAns ${ }^{\text {a }}$ |  |  |  |  |  |
| ${ }_{86}$ | 86 |  |  | Hatas6 |  |  |  |  |  |  |  |  |  |  | ImABrs6 |
| \％ | ${ }_{8} 8$ |  |  | Heatrs8 |  |  |  | Oin |  |  |  |  |  |  |  |
| － |  |  |  | ${ }_{\text {Heat }}^{\text {Heat }}$ |  |  |  |  | OinA ${ }_{\text {cos }}$ |  |  |  |  |  | $\xrightarrow{\text { max } \mathrm{max}_{50}}$ |
| 9． | 91 $\mathrm{Hffa}_{\text {m }}$ 91 |  |  | Heats ${ }^{\text {at }}$ |  |  |  |  |  |  |  |  |  |  |  |
| $\frac{92}{23}$ | 92pratare |  |  | $\mathrm{Ha}_{3} \mathrm{~S}_{5} \mathrm{~S}_{2}$ | Prean $\mathrm{S}_{\text {2，}}$ |  |  |  |  |  |  | $\mathrm{Dras}_{\text {ma }}$ |  |  |  |
| $\stackrel{93}{94}$ | － |  |  |  | Peceat |  | Masam3mat |  |  | ${ }_{\text {Triname }}$ |  |  |  |  |  |
| $\frac{95}{96}$ | ${ }^{9.5}$ |  |  |  |  | DreA $\mathrm{ma}_{\text {a }}$ S |  |  |  |  | TmaAcas |  |  |  |  |
| ${ }_{97}$ | ${ }_{97}{ }^{\text {P／ffacal }}$ | $\mathrm{XITAAQ}^{9} 7$ |  | ${ }^{\text {Hasasa }}$ |  |  |  |  |  |  |  |  |  |  |  |
| $\stackrel{98}{9}$ |  |  |  | ${ }^{\text {HaAa }}$ as8． |  |  | Masams， | Onilums ${ }_{\text {mad }}$ |  | TriAAmes， |  | Deasame |  |  |  |
| $\stackrel{\text { 900 }}{\substack{\text { 100 }}}$ | 迷 |  |  |  | Pisecarilo | Dreatas ${ }_{\text {dea }}$ |  |  |  |  | TraAas9 |  |  |  |  |
| －101 | ， |  |  |  |  |  |  |  |  |  |  |  |  | miniomer |  |
| 103 |  | $\mathrm{Xtramam}^{103}$ |  | Hatal $133^{\text {a }}$ ， | Pseatilo |  |  |  | Ohatali3 |  |  | － |  |  |  |
| （104 |  | $\chi_{\text {ratames }}$ |  |  |  |  |  |  |  |  |  |  | Ohas ${ }_{\text {cex } 14}$ |  |  |
| ${ }^{106}$ | 6， |  |  | Hatal 106 |  |  |  |  |  |  |  |  |  | $\mathrm{Tinab}_{\text {cat } 106}$ |  |
| －108 |  |  |  |  | Pecaral 108 |  |  |  |  |  |  |  |  |  |  |
| ${ }^{100}$ | 10， |  |  |  | Psecallo9 |  |  |  |  |  |  |  |  |  | makes 109 |
| $\frac{111}{111}$ |  |  |  | ${ }_{\text {max }}^{\text {Hatal }}$ |  |  |  | Oiniamlll | Ina $A_{\text {clill }}$ | TiniAcalll | TruA $A_{\text {cell }}$ |  |  |  |  |
| $\frac{112}{113}$ |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 114 |  |  |  | Hasen 114 |  | Drea $\mathrm{Acx}^{\text {cli }}$ |  |  |  |  |  |  |  |  |  |
| $\frac{115}{116}$ |  |  |  |  |  |  | Matallis |  |  |  | rindacl16 |  |  |  |  |
| $\frac{117}{118}$ |  |  |  | $\mathrm{Ha}_{4} \mathrm{a}_{10} 17$ |  |  | Msatar 117 |  |  |  |  |  |  |  |  |
| － 119 | （18） |  |  | ${ }_{\text {max }}^{\text {Hatan }}$ |  |  | Mantill ${ }_{\text {max }}$ | Oiliar 19 |  |  | Trantact 19 |  |  |  |  |
| $\frac{120}{122}$ |  |  |  |  | Pea， 121 | Dratamal |  |  |  |  |  |  |  | ${ }_{\text {mincem } 121}$ |  |
| ${ }^{122}$ | 22 | XTAA， 122 | Lneen 122 | Hatal122 |  |  |  |  |  |  |  |  |  |  |  |
| － |  |  |  |  |  |  | Matax 123 |  |  |  |  |  |  |  |  |
| ${ }^{125}$ | ${ }^{2.5}$ |  |  | ${ }^{\text {Hatat } 125}$ |  |  |  |  |  |  | TruA $A_{\text {ara }} 12 \mathrm{~S}$ |  |  |  |  |
| $\frac{128}{127}$ | 退 |  |  | ${ }_{\text {Hata }}{ }^{\text {Hat } 26}$ |  | Dreatack 27 |  |  | OhaA ${ }_{\text {a }} 126$ |  |  |  |  |  |  |
| ${ }^{128}$ |  |  |  |  | Preatal28 |  | Masat128 |  |  |  |  |  |  |  |  |


| 129 |  |  |  | Hasau 129 |  |  |  |  |  | TniA $A_{\text {cki } 129}$ |  |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| 130 | Hiraver $130_{\text {，}}$ |  | Lme $A_{\mathrm{u}} 133_{\text {dex }}$ |  | PseA， 130, |  |  |  |  |  |  |  |  |  |  |
| ${ }^{131}$ |  |  |  |  | $\mathrm{Psec}_{4 \times \mathrm{x}} 131$ |  |  |  |  |  |  |  |  |  | TruABex 131 |
| 132 | Hifak 132. |  |  |  | PseA，${ }_{\text {k }} 132.4$ |  |  | $\mathrm{OniA}_{\text {ck }} 132{ }_{\text {ded }}$ | Oha $A_{\text {kx }} 132_{\text {atad }}$ | TniA $A_{\text {cax }} 132$ ，${ }_{\text {add }}$ | TruA $A_{\text {ckil }} 132_{\text {asad }}$ |  |  |  |  |
| ${ }^{133}$ |  |  | Lme ${ }_{\text {k }}$ 1 133 | $\mathrm{HzaA}_{\text {a } 133}$ | PseeA $\mathrm{A}_{1 / 13} 13$ |  |  |  |  |  |  |  |  |  |  |
| ${ }^{134}$ |  |  | Lme ${ }_{\text {x } 11} 134$ | HsaA $\mathrm{A}_{1} 134$ | $\mathrm{Psec}_{\text {cel }} 134$ |  |  |  |  |  |  |  |  |  |  |
| 135 |  |  |  |  | PseA $A_{\text {cl }} 135$ |  |  |  |  |  |  | DreABscr 135 |  |  |  |
| ${ }^{136}$ | Hfrax． 136 |  |  |  | PreAk．136m |  |  |  |  | TniA $A_{\text {cl }} 136_{\text {m }}$ |  |  |  |  |  |
| 137 |  |  | LmeA ${ }_{\text {al } 137}$ | $\mathrm{H}_{5} \mathrm{~A}_{\text {a } 1.137}$ |  |  |  |  |  |  |  |  |  |  |  |
|  | $\mathrm{Hframax}^{138_{\mathrm{s}}}$ |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 139 |  |  |  | HsaAkl 139 |  |  | MsaA ${ }_{\text {a }} 139$ |  |  |  |  |  |  |  |  |
| 140 | Hiragk $140_{\text {ata }}$ | $\mathrm{XIFA}_{\text {a }} 140_{\text {and }}$ |  | Hsask． $14 a_{\text {cost }}$ |  | DreAAs．140 ${ }_{\text {ase }}$ | Msatas 140 ，max | $\mathrm{omia}_{\text {a }} 140_{\text {max }}$ |  | TniA $A_{\text {gr }} 140$ ，mata | $\mathrm{TruAA}_{\text {a }} .140_{\text {anad }}$ |  |  |  |  |
| PFC | Homshark A | Frog A | Coclacamth | Human A | Bichir A | Zetrafish AA | Striped Bass A | Nile Tilpria A | Medaka AA | Tetradon AA | Takifugu AA | Zebralish AB | Medaka $A B$ | Tetraodon AB | Takifug AB |
| 141 |  |  |  |  |  |  | － |  |  |  | ， | DreABsar 141 |  |  | － |
| 142 |  |  |  |  | PseAcl 142 |  |  |  |  |  |  | DreA $\mathrm{Agxs}^{142}$ |  |  |  |
| 143 |  |  |  |  |  | DreAA $A_{\text {cl }} 143 \mathrm{~mm}$ |  | $\mathrm{OniA}^{\text {a }} 1433_{\text {mad }}$ |  | $\mathrm{TniA}^{\text {A }}$ A． 143 lma |  |  |  |  |  |
| 144 |  |  |  |  | PseA $\mathrm{AlP}^{1} 144$ | DreA $A_{\text {a }} 144$ |  |  |  |  |  |  |  |  |  |
| 145 |  |  |  | $\mathrm{HsaA}_{\text {al }} 145$ |  |  |  | ${ }^{\text {oni }}{ }_{\text {a }} 14.145$ |  |  |  |  |  |  |  |
| 146 |  |  |  | $\mathrm{HsaA}_{\text {al }} 146$ |  |  |  |  | OhAAs．146 |  |  |  |  |  |  |
|  | Hframel 147 |  |  | $\mathrm{HsaA}_{\text {ala }} 147$ |  |  |  |  |  |  |  |  |  |  |  |
| 148 | Hff $A_{\text {cl }} 148$ |  |  | $\mathrm{H}_{5 a} \mathrm{asc}_{1} 148$ |  |  |  |  |  |  |  |  |  |  |  |
| 149 | Hfratil 19 | Xtram 149 | Lme $A_{\text {cl }} 149_{\text {dec }}$ | Hsa $A_{\text {cli }} 149$ | Pse $A_{\text {ckil }} 149_{\text {me }}$ | DreA $A_{\text {cl }} 149 \mathrm{Na}$ |  |  | Oha $A_{\text {cl }} 14 \mathrm{~m}_{\text {max }}$ | TniA $A_{\text {cli }} 149 \mathrm{mad}$ | TruA $A_{\text {ala }} 149 \mathrm{mad}$ |  |  |  |  |
| 150 |  |  | Lme $A_{\text {cl }} 150$ | HsaA ${ }_{\text {cl }} 150$ |  |  |  |  |  |  |  |  |  |  |  |
| 151 |  |  |  | $\mathrm{Hsa}^{\text {a }}$ ， 151 |  |  |  | $\mathrm{OniA}_{\text {a }} 151$ |  |  |  |  |  |  |  |
|  |  |  |  | $\mathrm{HsaA}_{\text {al }} 152$ |  |  |  |  |  |  |  |  |  |  |  |
| 153 |  |  |  | HaAakı 153 |  |  |  |  |  |  |  | DreABac153 |  |  |  |
| 154 |  |  |  | $\mathrm{HsaA}_{\text {a } 1.154}$ |  |  |  | ${ }_{\text {oniA }}$ ， 154 |  |  |  |  |  |  |  |
| 155 | Hffakl 155 |  |  | HsaAkı 155 |  |  |  |  |  |  |  |  |  |  |  |
| 156 |  |  | LmeAk．156 | HsaAk．156 |  |  |  |  |  |  |  |  |  |  |  |
| 157 | $\mathrm{Hffa}_{\text {k }} 157_{\text {k }}$ |  | ${ }_{\text {Lme } A_{\text {k }} 1577_{\text {cex }}}$ | ${ }^{\text {Hsa } A_{k 1} 157_{x}}$ |  | $\mathrm{DreAA}_{\text {ald }} 157_{\text {lue }}$ |  | ${ }^{\text {oni }} \mathrm{A}_{\mathrm{k}} 157_{\text {dex }}$ | ${ }^{\text {LuAAA }}$ ALI $157_{\text {luc }}$ |  | $\mathrm{TuAA}^{\text {a }} 157_{\text {dex }}$ |  |  |  |  |
| 158 |  |  |  |  |  | DreA Amm 158 | 将位品 |  |  | TniA $A_{\text {ali }} 58$ |  |  |  |  |  |
| 159 |  |  |  |  | $\mathrm{Psec}_{\text {a } 1159} 159$ |  | mmmmmom |  |  |  |  | DreABcal 159 |  |  |  |
| 160 |  |  | LmeA ${ }_{\text {Lum }} 160$ | $\mathrm{HzaA}_{\text {Lala }} 160$ |  |  |  |  |  |  |  |  |  |  |  |
| 161 | Hfatal161 |  |  | Hsa $A_{\text {ald }} 161$ |  |  |  |  |  |  |  |  |  |  |  |
| 162 |  |  | Lme $_{\text {AM1 }}$ 162 | HsaAtul 162 |  |  |  |  |  |  |  |  |  |  |  |
| 163 |  | ${ }_{\text {XIt } A_{\text {Lam }} 163}$ |  | HsaAtal 163 |  |  |  |  |  |  |  |  |  |  |  |
| 164 |  |  |  |  |  | DreA $A_{\text {am }} 164$ |  |  |  |  |  |  |  |  |  |
| 165 |  |  |  |  |  | $\mathrm{DreA}^{\text {amm }} 165$ |  |  | $\mathrm{OhAA}_{\mathrm{A}_{\mathrm{ml}} 165}$ |  |  |  |  |  |  |
| $\frac{166}{167}$ | ${ }_{\text {Hir }}$ |  |  | ${ }_{\text {HsaA }}^{\text {Hel } 166}$ |  |  |  |  |  |  |  |  |  |  |  |
| $\frac{167}{168}$ | Hira $\mathrm{A}_{1 / 167}$ |  |  | HsaAavil67 |  |  |  |  |  |  |  |  |  |  |  |
| 168 | Hira ${ }_{\text {ald }} 168$ |  |  | ${ }^{\text {HaA }}$ ati 168 |  |  |  |  |  |  |  |  |  |  |  |
| $\frac{169}{170}$ |  |  |  |  |  | $\mathrm{DreA}_{A_{\text {am }} 169}$ | vw,w,w, |  |  |  |  |  |  |  |  |
| 170 |  |  |  | $\mathrm{HSaA}_{\text {Lur }} 170$ |  |  |  |  |  |  |  |  |  |  |  |
| 171 172 |  |  |  |  |  |  |  |  | OhAA ${ }_{\text {as，}} 172$ | TniA $A_{\text {wil }} 172$ | TruA ${ }_{\text {wim }} 172$ |  |  | TniABas171 |  |
| 173 |  |  |  |  |  |  |  |  | $\mathrm{OLAA}^{\text {a }}$ M $1 / 2$ | $\mathrm{ThiAA}_{\text {Lu }}$ | TruA ${ }_{\text {M }} 1 / 2$ |  |  |  |  |
| 174 |  | ${\mathrm{XITA} A_{\text {LI }} 174}^{\text {a }}$ |  | HzaAalul7 |  |  |  |  |  |  |  |  |  |  |  |
| 175 |  |  |  | Hsa $A_{\text {ald }} 175$ |  |  | ，mmmmmom | OniA ${ }_{\text {all }} 175$ |  |  |  |  |  |  |  |
| 176 |  |  |  | HsaAtul76 | PseA，${ }^{\text {a }} 176$ |  |  |  |  |  |  |  |  |  |  |
| 177 | Hifa ${ }^{\text {a }} 177$ |  |  | HsaA An 177 |  |  |  |  |  |  |  |  |  |  |  |
| 178 |  |  |  | HsaAasl178 |  | ${ }^{\text {DreAA }}$／${ }^{178}$ |  |  |  |  |  |  |  |  |  |
| 179 | Hifa $\mathrm{A}_{\text {a }} 179_{\text {ate }}$ | Xtraiml 179, | Lme $\mathrm{A}_{\text {ama }} 179$ ， | Hsa ${ }_{\text {asal }} 179$ ， |  |  | 元 | $\mathrm{M}^{\text {OniA } A_{41} 179_{\text {，}}}$ | OLAAAm179 ${ }_{\text {en }}$ | TniAA ${ }_{\text {ana }} 179$ ， | TuAA ${ }_{\text {ama }} 179, \ldots$ |  |  |  |  |
|  |  |  |  |  |  |  | （tymm |  |  |  |  |  |  |  |  |
| 181 <br> 182 | Hifial $1811_{\text {cate }}$ |  |  |  |  |  | 䉼加加加 |  |  | TniA $A_{\text {us }} 181_{\text {daxa }}$ | TruA $A_{\text {ma }} 181_{\text {atate }}$ |  |  |  |  |
| 183 |  |  |  |  |  | DreA $A_{\text {am }} 183$ |  |  |  |  | TruA $A_{\text {m }} 183$ |  |  |  |  |
| 184 |  |  | $L_{\text {Lme }}^{\text {amm } 184}$ | HsaAtul 84 |  |  |  |  |  |  |  |  |  |  |  |
| 185 | Hifa $A_{\text {a }}^{185}$ |  |  | HsaAtul185 |  |  |  |  |  |  |  |  |  |  |  |
| 186 |  |  |  | ${ }_{\text {Hasaut }} 186$ |  |  |  |  |  |  | TruA ${ }_{\text {ma }} 186$ |  |  |  |  |
| 187 |  |  |  | HsaA ${ }_{\text {al } 187}$ |  |  |  |  |  |  | TruA ${ }_{\text {ma }} 187$ |  |  |  |  |
| 188 | Hira a $_{\text {a }}^{188}$ | ${\mathrm{XIFA} \mathrm{Lam}_{\text {I }} 188}$ | $L_{\text {Lme }} \mathrm{A}_{\text {Lu }} 188$ | ${ }_{\text {Hataial }}$ 188 |  |  |  |  |  |  |  |  |  |  |  |
| 189 |  |  |  |  | PseAmal 189 |  |  |  | Oha ALum 189 |  |  |  |  |  |  |
| 190 |  |  |  | HsaAas， 190 |  |  |  |  |  |  |  |  |  |  |  |
| 191 |  |  |  | HsaA AxI 91 |  |  |  |  |  |  |  |  |  |  |  |
| $\frac{192}{193}$ |  | ${ }_{\text {XIFALM }} 193{ }^{\text {asd }}$ |  |  | PseAul193 ${ }^{\text {a }}$ |  |  | $M^{\text {a }}$ |  |  |  |  |  |  |  |
| 194 | ${ }^{\text {a }}$ | ${ }^{\text {Premen }}$ | Lme $A_{\text {max }} 19 h_{\text {axe }}$ |  |  |  | （m） |  |  |  | ${ }^{\text {Trum }}$ |  |  |  |  |
| 195 | Ampr | Matad |  |  | PseAmil9 | drampad |  |  |  |  |  | $\mathrm{DreABgax}^{195}$ |  |  |  |
| 196 |  |  |  |  | PseA Al｜l｜ 196 |  |  |  | $\mathrm{OhAA}^{\text {amm }} 196$ |  |  |  |  |  |  |
| 197 |  |  |  |  |  | $\mathrm{DreAA}_{\text {Lum }} 197$ |  |  | Oha ${ }_{\text {asm }} 197$ |  |  |  |  |  |  |
| 198 | Hifatul 198 |  | $L_{\text {Lme }}$ | Hsa $_{\text {a }} 1989$ | Psea $_{\text {a }}^{\text {a }} 1989$ |  |  |  |  |  |  |  |  |  |  |
|  | Hfratul $199_{\text {mas }}$ | ${ }^{\text {Xtra } A_{\text {LI }} 199}$ |  | Hza $_{\text {Lut }} 199$ ， |  | DreA $A_{\text {LIM }} 199_{\text {vad }}$ |  | $\mathrm{m}^{\text {Oni } \mathrm{A}_{\text {cli }} 199_{\text {mat }}}$ | $\mathrm{OhAA}_{\text {lam }} 199_{\text {wad }}$ | $\mathrm{T}^{\text {ThiA } A_{\text {lal }} 199_{\text {ked }}}$ | ${ }^{\text {TruA } A_{\text {Lu }} 199_{\text {kad }}}$ |  |  |  |  |
| 200 <br> 201 | Hifama 201 | $\mathrm{XIFA}_{\text {amz } 201}$ | LmeAmz201 | HsaAms201 |  |  |  |  |  |  | TruAA，${ }^{\text {a } 200}$ |  |  |  |  |
|  | Hframe202 |  |  | HsaAmz22 |  |  |  |  |  |  |  |  |  |  |  |
| 203 |  |  |  | HsaAms203 |  |  |  |  |  |  |  |  |  |  |  |
| 204 |  | ${ }^{\text {XIta } A_{\text {a }} 204 \text { amad }}$ |  | ${ }^{\text {Hsa }}$ asm204med |  |  | 者 | $\mathrm{M}^{\text {Oniams } 204 \mathrm{mad}}$ |  | TniA $A_{\text {ses }}$ 204tasa | TruAA Ans204，ad | DreABar 204 mad | Ola ${ }_{\text {asas } 2044_{\text {c }}}$ | $\mathrm{TriABmaxamad}^{204}$ | ${ }^{\text {TruABsax } 204 \mathrm{tad}}$ |
| 205 |  |  |  |  | PseAns205 |  |  |  |  |  |  | DreABar205 |  |  |  |
| 206 |  |  |  |  | PseA，${ }^{\text {ck } 206}$ |  |  |  |  |  |  |  |  | $\mathrm{T}_{\text {miA } \mathrm{Bax}^{206}}$ |  |
| ${ }_{207}^{208}$ |  |  |  |  | PseAns207 |  |  |  |  | TniA Anv207 |  |  |  |  |  |
| 208 <br> 209 |  |  |  | ${ }^{\text {Hsa } A_{\text {was }} 208}$ |  | DreAA ${ }^{\text {w209 }}$ |  |  |  |  |  |  |  |  | $\mathrm{TruAB}_{\text {cav } 208}$ |
| 210 | Hfans 210 | Xtraum $210{ }_{\text {a }}$ | LmeAms210 ${ }_{\text {dec }}$ | Hsammer 210 ate | PseAswa 210 ， |  | 芷 |  |  |  |  |  |  |  |  |
| PFC | Homshark A | Frog A | Coelacamth A | Human A | Bichir A | Zetralish AA | Striped BassA | Nile Tilppia | Medaka AA | Tetradon AA | Trakifugu AA | Zetrafisis $A$ B | Medaka AB | Tetraodon AB | Takifuy AB |
| 211 | Hframe $211_{\text {lade }}$ |  |  |  | PseAms $21 l_{\text {wast }}$ |  |  |  |  |  | TruA Aw 211 ，mats |  |  |  | Truab ${ }^{\text {a } 211}$ |
| $\frac{212}{213}$ |  |  |  |  | ${ }^{\text {Psea } A_{\text {c }} 212}$ |  |  |  |  |  |  |  |  | 車事事事 |  |
| 214 |  |  |  |  | PseAs ${ }^{\text {a }}$ 214 |  |  |  |  |  | TruAAvo214 |  |  |  |  |
| 215 | Hffano 115 |  |  | HsaA ${ }_{\text {so } 215}$ |  |  |  |  |  |  |  |  |  | － |  |
| 216 |  |  |  |  |  | DreAAso216 |  |  | Oha ${ }_{\text {ans } 216}$ | TriA ${ }_{\text {mse }}$ 216 | TruA ${ }_{\text {ms } 216}$ |  |  | ＋ |  |
| 217 <br> 218 | Hfravo218 |  |  |  |  | DreAAso217 |  |  | Oha $\mathrm{soz}^{217}$ |  |  |  |  |  |  |
| 219 | Hirasoc 19 |  |  | $\mathrm{HsaA}_{\text {so }} 19$ |  |  | mmmmm | Oni ${ }_{\text {va } 219}$ | Oha $\mathrm{Avol}^{219}$ | TniA $A_{\text {so }}$ 219 |  |  |  |  | $)^{+}{ }^{+}$ |
| 220 |  |  |  | $\mathrm{HaA}^{\text {Haso } 220}$ |  |  |  |  | Oha $\mathrm{Na}_{0} 220$ |  |  |  |  |  | － |
| 221 |  |  |  | HsaAso221 |  |  |  |  |  |  |  |  |  |  |  |
| $\stackrel{223}{223}$ |  |  |  |  | $\mathrm{PSEA}_{\text {aso }} 222$ | $\mathrm{DreAANow}^{223}$ |  | ${ }^{\text {ania } 222}$ | ${ }^{\text {Oha } A_{\text {a }} \text { O22 }}$ | TinA $\mathrm{A}_{\text {so }} 22$ | TruA Avo 22 | 比地地址 | ＋ | ＋ | ＋ |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |










| Oreochromis niloticus Nile tilapia Hox Aa |  |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: |
| PFC Dist to $3^{\prime}$ gene |  | Length Sequence |  |  |  |
| Oni $\mathrm{A}_{\text {Ac }} 3 \mathrm{O}_{\text {ab }}$ | 1,073 | 35 tggctagacagcaacattcaacttgaccttgccct |  |  |  |
| OniA $A_{\text {c }} 36$ | 10,037 | 24 CcGGgtcttatcgacgiggaigai |  |  |  |
| OniA ${ }_{\text {cE }} 42$ | 9,408 | 23 attattattattattitctittg |  |  |  |
| OniA $\mathrm{CE}^{54}$ | 3,540 | 38 GCCTGTAGTAAAGCTTTGAGCTtTTTCTtTTCCTTGAA |  |  |  |
|  |  | GCTATAAAAACCCAGGTCTGTGCAGTGCTGGCTTTGGTTTTCCTGG GCATCCTGTCCTCAATTACAGCTTAAAAGCTTCGGCACAACTCTTA |  |  |  |
| Oni $\mathrm{A}_{\mathrm{cE}} 57_{\text {bc }}$ | 2,236 | 120 gaittigtctgaattgaccgagaggcga |  |  |  |
|  |  | TGTCAATTTCTGCCGTGTGGTCGGTCACGTGACCTCCTCCTCCGTG gagtgaatggagatgactetccacgtcagcctacgtctcccanttt |  |  |  |
| OniA $A_{\text {cE }} 60_{\text {abcd }}$ | 206 | 118 ctgctagigaacctacticanagag |  |  |  |
|  |  | CCTAATTACGGGACATCCTCCCCGTTGCCGCAGCAACGCGGCCAT <br>  |  |  |  |
| Oni $A_{E F} 65_{\text {bcd }}$ | 3,627 | 128 TGCAATAAACCGTCTGAGACCCAAGGITATTAACTGTG |  |  |  |
|  |  | ctctgaantttatgcacacaggitttatataanattaaganagtg |  |  |  |
| OniA $A_{\text {EF }} 72$ | 1,161 | 50 сСтT |  |  |  |
|  |  | GGGAGGGACTGCCCATCTTCCAGCTTTTCCATTGGTTTCACAGTCT GCTCGGCGGAGAGGGGGTGCCTCTAATCATATCCAGCATGTTTTGC |  |  |  |
| OniA $A_{\text {EF }} 75_{\text {acde }}$ | 150 | 137 ACAAGAAATGTCAGCCAGAAAGGGCTACCTTCTCTCTTCGCCAAA |  |  |  |
| OniA $\mathrm{FGG}^{\text {76 }}$ | 3,273 | 12 agtittatgact $\quad$ agGTagtticatgitgitggagtccattictanctctgcaacatga |  |  |  |
| OniA $A_{\text {FG }} 799_{\text {bde }}$ | 2,879 | 69 a actgtcttanttgccleagtta |  |  |  |
| OniA Fg $^{8} 8$ | 1,374 | 20 angaattatgcatttaatt |  |  |  |
|  | 1,721 | atacagtatattaaatgiatcctgctgcaggccattccccgcgeca 58 ttTCcttttana |  |  |  |
| OniA FG88 $^{\text {8 }}$ |  | CACGTGTGTCTGCTACCCAATGGCATGGCAGCCTGTCTCCCCATTA CTTTCCCACTGTAGTTCTCTGTGGAGCGAAGTTGCTACTTGATTTCT |  |  |  |
| OniA $A_{\text {FG }} 93_{\text {abdet }}$ | 219 | 127 CCACATTGTTATTTTGTGAGGCTGGGTTTACTGC |  |  |  |
| OniA gk $^{\text {9 }}$ | 10,308 | 19 actacctitatattgcaca |  |  |  |
| OniA $\mathrm{Gk}_{\text {g }} 96$ | 10,146 | 19 Ctatattigitgictatct |  |  |  |
| OniA ${ }_{\text {GK }} 98_{\text {abcd }}$ | 9,217 | tgitagctgtatacagccatanangacaattaccgctatancctit 73 TATGGGGTGCAAAGCGCTGCGAGGCGA |  |  |  |
|  |  | GCAGATGGACCCAAACTTCAAAGACTCGGCCAGAGACAGTGCAA taAAACGCCTGGTCTGCTATACTGTCTGGCATTCCAGTTTTAATGG CTTTATGGCCGTCCAGACACAATTAGGCCGCTTCCAGAATGGCAC CCATTTGTTTTTTCTTCTCTTTCTGTGAGACTGCGGTCTGGACAAAA gGcccGagcgaaatgatcagttitattgGattctcctcgacggag |  |  |  |
| OniA $A_{\text {GK }} 103_{\text {bcdef }}$ | 8,610 | 289 ACGCGCAGGACTCACGGTCAtttgGag |  |  |  |
| OniA ${ }_{\text {GK }}{ }^{111}{ }_{\text {b }}$ |  | gittggtataiatctagggigtattgctgicatctatcacactacct |  |  |  |
|  | 6,657 | 84 CGTAAAAACGACACTGAGGATTCTGGGCCAACAAATC |  |  |  |
|  |  | ATGGCGCCTGACGCTGACCTGCCCTCCTCCCTCCCTCСTTTTTCTGT СТСТСТСТСТССССССGТСССТСТСССТССАGСTCTGGGCCATAAA TCCGTTGTTGTTTATGAAAATTTACAACATAGCCATACAAGTTTAC GACGCGGCCCGGGTTCCCATTGGCCGCAGCTTGTCACGTGGCTGG |  |  |  |
| OniA AK $^{1119_{\text {abc }}}$ | 3,871 | 200 gagccatgacatgai |  |  |  |
|  |  | TGGCGCACCACGACAATCCAAAACACCCAAAGCCCCGGCTCAGA gittgccetagccacgagcgcaggaanagcgcgcaccccttatat CCCACCCAGTATTTCCTGCGTGCACGAGTTTACCTCTGGAGGTCAC cGagCaggatttacgactggtcancaaangcacgtgattcttcge Cataccccatatttgggtgcctacgtangagagaatcangtccat |  |  |  |
| Oni $A_{\text {GK }} 132_{\text {abcd }}$ | 484 | 392 GTCCCACTCATTTCCATAATTCATCATAAA |  |  |  |
|  |  | aAACTTTATTAGGGCGGTTTCTGGCTCTCTGACATTTGGGTGCTAA ATGAATGGGGGGTTTGTCTATGAATTAGATCGTAAAAATCATCCGG AGAGCGGCCAGATAGGCTCACTGGCCATAAACGGCAGTATATGGC |  |  |  |
| OniA ${ }_{\text {kL }} 136_{\text {abc }}$ | 7,537 | 205 acataicatataatcgiactga |  |  |  |
| Oni $A_{\text {KL }} 140_{\text {abcole }}$ |  | GGTtCAtCTGGAGGTCAGCCACAGCACCAAGATAAATCTGCATCC |  |  |  |
|  | 5,596 | 84 TCTCTGAGCCACCAGCAGAGCTCGCTTTAGGCCAAGTTC |  |  |  |
|  |  | GGTCAAAAGTTTACGCGCTGAACAACTCTCCGTCATCTGTTCACTC GGAGCCTGATGCCAGCCTTATAATAGCGATCTTGACTCTCCACAC AAAAGACAGAATAGCTTTGCATTACATATGTTGCATGGTGCACTCC AGGTGAACCCTGGATGCGCAGTGACCTCGCGTTTGAAACCGGGGG AGGAGACCCCCCACCCGTCCCCAGCAACAAGATTGAGTGGTTGGC |  |  | AAGGCGCTACTTCATTTGCATAATTTTTCTTGTCTATGGTGACGCTC TGGTTCGGGGCAGTTCACTGGTGACTGCATGGTACTAGTGAAGGAT |
| OniA ${ }_{\text {kL }} 143_{\text {bcd }}$ | 4,958 | 311 GTtteattagggcgactgattggtaat |  |  | CaCATACACCAGCATAGCCTTGGCCGACTTTAATATTGCTATGAA gCCCTGGTCATTGCTCTATAGCAGGACGATACATTCAATCTTGGGG |
| OniA ${ }_{\text {Kı }} 145$ | 2,825 | 21 actgantactttgacattta | Oni $A_{L M} 193_{\text {abod }}$ | 2,455 | 201 ccagatangtcttatct |
|  |  | TCCAAGTCCGGGGTGAACCCCGGGTCACTGCGTCTAACAGATATG AAAATGTCGCCTCTTTGGAAAAGGGCACGCCTTGTTGTTTAACAA agactgTcantggciangattantcagaiacanaitgaanacgg |  |  | atcgatttcgtcgctgantgaganaatattgcttgatgctctgcat tcgtcgtangaggatangataigcaggccaggantaggctccctc gGtTGTAAATGGCTGAGTTTGTATGTCGCGCGGTGATTTATCACCG |
| Oni $A_{\text {kı }} 149_{\text {bode }}$ | 1,758 | 169 tgicactatggagtcaggcagaagitatctctgr | OniA LIL $^{194}{ }_{\text {acd }}$ | 2,028 | 169 tatgacttagatctcgattcaggangagttca |
| OniA ${ }_{\text {kı }} 151$ | 1,254 | 45 tTAttattactattattattattattattattataggtttcttg |  |  | TGTCAGGGCTGCATTGAAAAGTAAGAGGGATCGCCACCATTTCTTC TCCTCACAGTGCTCTGTAACCCTAGGTTCACCCAAGGAGGCCAT |
| Oni $A_{\text {kL }} 154$ | 497 | 17 ССтTтTСтTтTCTITTC |  |  | tgGcGGagaggcgicacgigaccacgggatgccantgitattcta CAAGGGTGTCAAGACCCTGTCAGTTTCTGAAATAAATATTGGGAA |
|  |  | cCattggitcctgittacatgatgcclacaggacacgccgtgattg | OniA $A_{L \mathrm{LL}} 199_{\text {bcd }}$ | 190 | 184 AC |
|  |  | gTGGCTCTTCACACGTGACCAGGCAACTTTGTACATTTGACAGGGA gTaGgagggttttgtgangatcagaanaicgacagcgcgatana | Oni $\mathrm{A}_{\text {мn }} 203$ | 2,658 | 45 cagagacacagctggagctaaaigactacactttggatgtgcaat |
| OniA $A_{\text {kL }} 157_{\text {abc }}$ | 164 | 164 aAttagtattgttccacticacaiatta |  |  | agaigccttanatgtgttgtaaggcaccgagctgtcagaccttt |
|  |  | tTAATGATTCACGGGCTCAAATAAAAGGGATtTAAGTTGACGTTGC 57 GTCACGTGAGC | Oni $A_{\text {mN }} 204_{\text {abocd }}$ | 1,720 | 91 gGcgagrangattgatcgcgcgcagccticcaggactctttgtt |
| OniA ${ }_{\text {Lm }} 172$ | 5,729 | 57 Gtcacgigac | Oni $A_{\text {MN }} 216$ | 1,686 | 25 GTCAGACCTTTTGGCGagTAAGATT |
| OniA Lm $^{173}$ | 8,368 | 16 tcctatggacaicaig |  |  |  |
| Oni $\mathrm{A}_{\text {LM }} 175$ | 6,224 | 24 CaAttagGactigggagcgatatt |  |  | tgTanatatanacagtcgiccccccccagctgagcgaggcgitct |
| OniA $\mathrm{LLM}^{179} \mathrm{~g}_{\text {ab }}$ | 5,711 | aAataAaAGGGAttTAAGTtGACGTtGCgTCACGTGAGCGCGGCG 53 cataatac |  |  | tCACCAGAGTTTTGGATCAATCAGGCAGACAGTGGCTTCTTTTGAT taAACCCCAAATTGTCATTGGGCAGAGGTAATCATGTGACAGGCA attcgatccantticanccttgtctccatcaattcantagttant |
|  |  |  | Oni $A_{\text {MN }} 211_{\text {abocdet }}$ | 344 | 213 Agcagctcgatccceatacgaccgtaitcag |
|  |  | CGGTCACGGACGGATATGTTTTTTCACGGCGGACGGAGTCAGAGCT | OniA ${ }_{\text {No }} 212$ | 3,601 | 16 tacaattancttgita |
| OniA L $_{\text {LM }} 181_{\text {abode }}$ | 5,469 | 135 ttcattactgicgigagttattgctgcgcaaggcagaggtcag | OniA no $^{213}$ | 3,472 | 20 anaataittttanaangta |
| OniA ${ }_{\text {LM }} 190$ | 3,178 | 13 GGCtttctttttg | OniA No $^{219}$ | 2,919 | 30 ctcatanatcactccgiggcatgaatgaga |
| OniA ${ }_{\text {LM }} 191$ | 2,978 | 37 Ctcagctattiganagcacaactatatatacatatat | OniA ${ }_{\text {No }} 221$ | 784 | 30 GAAGTтTTCTGTCTTTTGСттстсстTCAC |
| OniA LM $^{192}$ | 2,438 | 28 GCataattittcttgtctatggtgacgi | $\mathrm{OniA}_{\text {No }} 222$ | 282 | 36 gcgicaccttgatcgacgagtgcttggaattaait |




| Takifugu rubripes Japanese pufferfish Hox Aa |  |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: |
|  | Dist to 3' gene Length Sequence |  |  |  |  |
|  |  | TTATTGGCCTCCCCTTTCGGGTTTGCGGGTTACGTGG |  |  |  |
|  |  | aAaCCCTGCatctttggcgcatgcanatttacatcc |  |  |  |
|  |  | agGctgatatgctatgtgagggactcattatccaag |  |  |  |
|  |  | TGGAGCAGAAACATTCTTGGTTAGATTTACCACAGC |  |  |  |
|  |  | gaggctccagccgacaccgigitcantggagagac |  |  |  |
| TruAA $A_{\text {c }} 5$ | 7,175 | 193 acagctctacagc |  |  |  |
| TruAA ${ }_{\text {AC }} 1$ | 5,423 | 16 Ctgtccttgtacgect |  |  |  |
| TruAA ${ }_{\text {AC }} 8$ | 1,704 | 34 ganaahgagangtcatatcancagtanatancai |  |  |  |
| TruAA ${ }_{\text {AC }} 14$ | 625 | 15 actganamangaat |  |  |  |
|  |  |  |  |  |  |
|  |  | TAATGTCCTTCAATCGCTCTCAATGGGGCAGGAATT |  |  |  |
|  |  |  |  |  |  |
|  |  | gTtCctttaangcctgaattgcccgacagcctggat |  |  |  |
|  |  | GGCTAGACGGCAACTTTCAACTTGACCTTGGCCTCC |  |  |  |
|  |  | agccgcgicctatggaggaahaaagcagtattttca |  |  | AAACTTTATTAAGGCCGATTCTGGGTCTGACATTTG GACGCTAAATGAATGGGGGGTTTTGTCTATGAATTA |
| TruAA ${ }_{\text {Ac }} 21$ | 1,030 | 235 GTGtttgcatcctatanaca |  |  | GACGCTAAATGAATGGGGGGTTTTGTCTATGAATTA |
|  |  | tGgctagacgicanctitcaactigaccttggcctc |  |  | ctcactggccatanacggtcacgigctagccattaa |
| TruAA ${ }_{\text {Ac }} 3 \mathrm{O}_{\text {abc }}$ | 888 | 42 cagccg |  |  | agtangitttatggittigggaagtigacagtatatt |
|  |  |  | TruAA ${ }_{\text {KL }}{ }^{136}{ }_{\text {abc }}$ | 3,673 | 204 gcacatancatataatcgcactga |
|  |  | CTtTGCATAGTCGGGTCACGTGGTACAACCCTGGCC |  |  |  |
|  |  | a thatggcgegtccteggcgetcctgtccacgica |  |  | gGttcatgtgalagtcatctccgacacgangatana |
|  |  | GAGTGGCAATACTCCCTGGGAACCAGTATCTCTTTT | TruAA ${ }_{\text {KL }} 140_{\text {abcd }}$ | 2,046 | 63 tctgcatcctctcagagctaccagcag |
|  |  | ttcagtccctggactttitanaanagagccacangc | KL ${ }^{\text {abcd }}$ |  |  |
| TruAA ${ }_{\text {AC }} 31_{\text {abder }}$ | 165 | 156 CTCAATGTTGGA |  |  | TCCAAGTACGGGGTGAACCCCAGGTCAGTGCGTCTA |
| TruAA ${ }_{\text {cE }} 48$ | 7,169 | 26 tantttagttctatggttgtaggana |  |  | ACAGATATGAAAACGTCGCCCCTTAGAAAAATGGC |
| TruAA ${ }_{\text {cE }} 53$ | 6,132 | 29 ttaanatantgcatgittitahcanaiat |  |  | GATTAATCAGAAACAAAATGGAAGCAGTGTCACTTT |
| TruAA ${ }_{\text {cE }} 55$ | 2,572 | 23 atanacgaccanccaggattana | TruAA kL $^{149_{\text {bcd }}}$ | 1,641 | 161 TGGgTCAGGCAGAAGTTA |
|  |  | GCTATAAAAACGCAGGTCTGTGCAGAGCTGCCTTTG |  |  |  |
|  |  | GTTTTCCTGGGCATCCTGTGCTCAATTACAGCTTAAA |  |  | CCATTGGTTCCTGTTTACATGATGCCCACGGGAGGC |
|  |  | agcttcagcacaactcgtaganttigtctgattga |  |  | gcGgtgattgatggcttticacacgigaccaggcas |
| TruAA ${ }_{\text {cE }} 57_{\text {bc }}$ | 1,889 | 120 ctgagaggcga |  |  | CTITGTACATTTGACAGGGAGTAGGAGGGTTTTGTG |
|  |  | TGTCAATTTCTACCGCGCGGTCACGTGATCTCCTCCT | TruAA ${ }_{\text {KL }} 157_{\text {abc }}$ | 164 | GaGATCAGAAAAACGACAGCGCGATAAAAATTAGT 164 ATTGTTGCACTTCACAAATTA |
|  |  | CCATGGAGTGGATGGAGATGGCTCTCCACGTCAGCT taCGTCTCCAAATTTCAGCATAGCAAACCTGCTTGA |  |  |  |
| TruAA ${ }_{\text {cE }} 60_{\text {abcd }}$ | 203 | 114 Aagag | TruAA ${ }_{\text {LM }} 172$ | 4,830 | 57 TTGACGCTGCGTCACGTGAGC |
|  | 2 |  |  |  | afataaakggattiangitgacgctgcgicacgig |
|  |  | CCTAATTACGGGACATCCTCCCTGTTGCCCCAGCAA | TruAA ${ }_{L M} 179_{a b}$ | 4,812 | 53 agcggggcgcatantac |
|  |  | cgiggccatanaagtcgtctgagagtctgcgacatt |  |  |  |
|  |  | tgtacaattggagtgcagtgcateanccgtctgag |  |  | ctgtcacgaacgeatatgcttgitcaacgegaccg |
| TruAA ${ }_{\text {EF }} 65_{\text {bcd }}$ | 3,300 | 128 Aaccanggttattanctgig |  |  | agtGgacticgagaghatggattitattitcaggtat |
| TruAA ${ }_{\text {EF }} 71$ | 1,325 | 15 tttattgcgasana |  |  | ttccgccaattgitcattaccaccgigagitattgct |
|  |  |  | TruAA ${ }_{\text {LM }}{ }^{181} 1_{\text {abcde }}$ | 4,567 | 128 GCAGGAGGCAGAGGTCAG |
|  |  | GGGAGGGACCGCTTATCTTCCAGTATTTCCATTGGTT |  |  | AGCAGTGAACGTGTTGTGATTGTGTCGCTCCGCCGG |
|  |  | TTACAGTCCGCACAGGGGAGAGGGGGCGCCTCTAAT |  |  | GGAAAACGAGCATATAATGGAAGACGGAGTGCCAT |
|  |  | CATATCCAGCATGTTTTGCACAAGAAATGTCAGCCA |  |  |  |
| TruAA ${ }_{\text {EF }} 75_{\text {acde }}$ | 150 | 137 GAAAGGGCTACCTTCTCCCCTCGCCAAA |  |  | TCGCTTGATTTATGGGCGCGGGGACTTGGGGAAGAT |
|  |  | GACTGTCGAGTGGTTTAGGTAGTTTCATGTTGTTGGG | TruAA ${ }_{\text {LM }} 183$ | 3,996 | 171 ggtgigcgagacctantgactattcgat |
|  | 2,700 | GTCCATTTCAAACTCTGCAACATGAAACTGTCTTAA <br> 85 TTGCCCCAGTTA | TruAA ${ }_{\text {LM }} 186$ | 3,871 | 13 cgaggacttggga |
| TruAA ${ }_{\text {FG }} 79_{\text {abde }}$ | 2,700 |  | TruAA ${ }_{\text {LM }} 187$ | 3,520 | 30 GTtTTGTTCATGTATGGGCGCCGTTGGAAA |
|  |  | CACGTGTGCGCGCTGCCCAATGACATCGCGCCTTGA |  |  |  |
|  |  | CTCCCCATTACAAGCCCACTGTAGTTCTCTGTGGGG |  |  |  |
|  |  | CCAAGTTGCTACtTGATtTCTCCACATTGTtattitg |  |  | tgacgetcaggitcgaggiagttactgctgtctac |
| TruAA ${ }_{\text {FG }} 93_{\text {abcdef }}$ | 212 | 127 gagactgtgtttactgc |  |  | gTGGTACTGGTGAaGGATCACATAGCCCAACATAG |
|  |  |  |  |  | CCTTGGCTGAGTTTGATATTTCCATGAAGCCCTGGCC |
| TruAA Gk $^{\text {9 }}$ 9 | 7,934 | 30 tttatttattanatanaacanattatatct |  |  | attectccttagcatgacgatacattcantctcgag |
|  |  |  | TruAA ${ }_{\text {LM }} 193_{\text {abd }}$ | 2,012 | 199 Cctagataictctiatct |
|  |  | atanaagacaattaccgicatanccttttatggagt |  |  |  |
|  |  | gCaAagcgctgcgaggcgagaggacacanamcaa |  |  | atcgatticgtcgctgatcagaiantatcgccagg |
| TruAA ${ }_{\text {GK }} 99_{\text {bcde }}$ | 7,590 | 98 AAAAAGACATCGAGAACGACGACAGCTG |  |  | TGCCCTGCATTGGTCGTCAGAGGATCAGGTAAGCAG |
| TruAA ${ }_{\text {gk }} 100$ | 6,809 | 20 CCtTTGCTTAGCCAGTCCTA |  |  | GCCAGAAATAGGCTCCCTCGGTTGTGAATGGCGGAG tTTGTGTGTCGTACGGTGATTTATCACCGTATGACTT |
|  |  |  | $\operatorname{TruAA}_{\text {LM }} 194_{\text {abcd }}$ | 1,591 | 169 agatctcggttcaggangagttca |
|  |  | gCagatggactcaancttcaangacgeccagagac |  |  |  |
|  |  | AGCGCAATAAAAACGCCTGGTCGGCTGTACTGTCTG |  |  | tgTCagccctgtattganaigtangatgantcgcca |
|  |  | GCATTCCAGTTTAAATGGTTTTATGGCCGTCCAGAC |  |  | ССАТТTСТТСТССТСАСАGТGСТTСTTGTAACCCTAG |
|  |  | ACAATTAGCCCGTTTCCAGAATGGCACCCATTTGTT |  |  | gttcaccegaggaggccattggaggagaggcgtca |
|  |  | тTTTCTCCTCTTTCTGTGAGACAACGCTCTGGACAAA |  |  | cgTGAACACGGGGTGCCAATGTTATTCTACAAGGGT |
|  |  | agGCCCagcgaanatgatcagttteattggattcce |  |  | gTCAAGACCCTGTCAGTtTCTGAAATAAATATTGGG |
|  |  | CGACGGGGACGCGCATGACTCGTGGTCATTTGTATA | TruAA ${ }_{\text {LM }} 199_{\text {bcd }}$ | 190 | 184 AAAC |
| TruAA ${ }_{\text {Gk }} 103_{\text {bccef }}$ | 7,098 | 285 GAG |  |  |  |
|  |  | GTTTGGTGTAAATCTAGGCTGTATTACTGTCATATAT | TruAA ${ }_{\text {Mn }} 200$ | 3,053 | 34 Cattccatgctgctccagttcgangaiatanata |
| TruAA ${ }_{\text {GK }} 111_{\text {b }}$ |  | CAAGCTACCTGGTAAAAACGACACTAAGGATTCTG |  |  | agaigccttaatgtgitgcgaggacaccgagctat |
|  | 5,556 | 83 GCCaACAAATC |  |  | Cagacctittggcgagtaagattgatcgcgcacagg |
|  |  |  | TruAA mı $^{204}{ }_{\text {abcd }}$ | 1,431 | 91 CTTCCAGCACTCTTTGTTT |
| TruAA ${ }_{\text {gk }} 116$ | 4,073 | 35 TGTtTTATATCAAAATGTCTAAAAAAAAAAAAAGT | TruAA ${ }_{\text {мN }} 216$ | 1,397 | 25 GTCAGACCTTTTGGCGAGTAAGATT |
|  |  | ATGGCGCCCGGTGGTGGGTGACCCCCCCTCTTCCTT |  |  |  |
|  |  | СТСТСТССТССАСССАGТTСТСАССАССТСТСССТСС |  |  | TGTAAATAAAAGCAGTCGTCCCAGCTGAGCGAGGC GATCTTCATCTGAGTTTTTTTTGGATCAATCATGCAG |
|  |  | gGCGCTGGCTCATAAATCGGCTGTtGittatgana |  |  | GATCTTCATCTGAGTTTTTTTTGGATCAATCATGCAG |
|  |  | TTTACAACACAGCGACGTAACTTTACGAGCGGACTC |  |  | GGGCagaggtaatcatgigacagcctattcgatcea |
|  |  |  |  |  | attTCAACCTTGTCTCCATGAATTCAATAGTTTAATA |
| TruAA ${ }_{\text {GK }} 119_{\text {abc }}$ | 3,321 | 197 GAGCCGTGAACATGAA | TruAA MN $^{211}{ }_{\text {abccete }}$ | 332 | 212 GTAGCTTGGTCCCCACACGACCATAATCAG |
| TruAA ${ }_{\text {Gk }} 125$ | 3,254 | 15 ccctecgacgetgac |  |  |  |
|  |  |  |  |  | agaanactanattganattagtagagtamatagag |
|  |  | tggcgegccacgacantacaanacangcgagttt |  |  | agccgtatgcaanttictcancaggitgccantgan |
|  |  | tCCTGCCTTTGAGCGCACGGAGGCGCGCACCCTCCA |  |  | tataatttgtatantcggatggcactgtgatgacac |
|  |  | CATCCCACCCAGTATTTGCTCTGTGCATGAGTTTACC |  |  | agGtttcactitttacaattcttttaattgcattgia |
|  |  | tctggaghtcaccaggcaggatttacgactgatcaa |  |  | aAtcttantgicgtacatcgatatataggatgcata |
|  |  | CaAamgcacgtgattcaccggcgiaccccatattig |  |  | TAAAGTAAATACTAAGATAAAAATCTAAGAACGCT |
|  |  | GTTGCCTACGTAAGAGAGAATCAAGTCTATGTCCCA | TruAA ${ }_{\text {No }} 214$ | 2,735 | 222 agcatt |
|  |  | ctCatticlataattcatcataanttgtgcaaggit |  |  |  |
| $\operatorname{TruA~}_{\text {GK }} 132_{\text {abcd }}$ | 376 | 374 GCT | TruA ${ }_{\text {No }} 222$ | 284 | 36 Gcgitacctigatcgacgagcgectagaitteanat |




| PFC | Frog B | Coelacanth B | Human B | Bichir B | Zebrafish Ba | Medaka Ba | Tetraodon Ba | Zebrafish Bb | Medaka Bb | Tetraodon Bb |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  |  |  |  |  | $\mathrm{DreBA}_{\text {Ac }}{ }^{1}$ |  | $\mathrm{TniBA}_{\text {cc }}{ }^{1}$ |  |  |  |
|  |  |  |  | $\mathrm{PseB}_{\mathrm{AC}^{2}}$ |  |  | $\mathrm{TniBA}_{\text {AC }}{ }^{2}$ |  |  |  |
|  |  |  |  | PseB $\mathrm{BaC}^{3}$ |  | $\mathrm{OlaBA}_{\text {Ac }}{ }^{3}$ |  |  |  |  |
|  |  |  |  | PseB $\mathrm{Ba}^{4}$ |  |  | $\mathrm{TniBA}_{\text {Ac }}{ }^{4}$ |  |  |  |
|  |  |  |  |  |  | $\mathrm{OlaBA}_{\text {cc }} 5$ | $\mathrm{TniBA}_{\text {cc }} 5$ |  |  |  |
| 6 |  |  |  |  | DreBA $_{\text {fG }} 6$ | $\mathrm{OlaBA}_{\text {cG }} 6$ |  |  |  |  |
| 7 |  |  |  |  |  | $\mathrm{OlaBA}_{\text {c6 }} 7$ | $\mathrm{TniBA}_{\text {cc }} 7$ |  |  |  |
| 8 |  |  |  |  | DreBA ${ }_{\text {cr }} 8$ |  | TniBA ${ }_{\text {cG }} 8$ |  |  |  |
| 9 |  |  |  |  | DreBA ${ }_{\text {cF }} 9$ |  | TniBAcc9 |  | H |  |
| 10 |  |  |  |  | $\mathrm{DreBA}_{\text {cr }} 10$ |  | $\mathrm{TniBA}_{\text {cG }} 10$ |  |  |  |
| 11 |  |  |  |  | DreBA $\mathrm{cF}^{\text {F }} 11$ |  | $\mathrm{TniBA}_{\text {cG }} 11$ |  |  |  |
| 12 |  |  |  |  | DreBA $\mathrm{CF}^{\text {cF }} 12$ | OlabA ${ }_{\text {cG }} 12$ |  |  |  |  |
| 13 |  |  |  |  |  | OlabA ${ }_{\text {cG }} 13$ | TniBA ${ }_{\text {cG }} 13$ |  |  |  |
| 14 |  |  |  |  |  | OlabA ${ }_{\text {cG }} 14$ | $\mathrm{TniBA}_{\text {cG }} 14$ |  |  |  |
| 15 |  |  |  |  |  | $\mathrm{OlaBA}_{\mathrm{cG}^{1} 15}$ | TniBA ${ }_{\text {cG }} 15$ |  |  |  |
| 16 |  |  |  | $\mathrm{PseB}_{\text {cr }} 16$ |  | OlabA ${ }_{\text {cG }} 16$ |  |  |  |  |
| 17 |  |  |  | $\mathrm{PseB}_{\text {cr }} 17$ | DreBA ${ }_{\text {cF }} 17$ |  |  |  |  | H |
| 18 |  |  |  | $\mathrm{PseB}_{\text {cr }} 18$ |  | OlabA ${ }_{\text {c6 }} 18$ |  |  |  |  |
| 19 |  |  |  | $\mathrm{PseB}_{\text {cr } 19}$ | DreBA ${ }_{\text {cF }} 19$ |  |  |  |  |  |
| 20 |  |  |  | $\mathrm{PseB}_{\text {cr } 20}$ | DreBA $\mathrm{cr}^{2} 20$ |  |  |  |  |  |
| 21 |  |  |  | $\mathrm{PseB}_{\text {FH }}{ }^{21}$ |  | $\mathrm{OlaBA}_{\mathrm{cc}^{2} 21}$ |  |  |  |  |
| 22 | $\mathrm{XtrB}_{\mathrm{cG}} 22$ |  | $\mathrm{HsaB}_{\mathrm{cc} 2} 2$ |  |  |  |  |  | ( |  |
| 23 |  | $\mathrm{LmeB}_{\text {cr }} 23$ | $\mathrm{HsaB}_{\mathrm{cc} 2} 23$ |  |  |  |  |  |  |  |
| 24 |  | $\mathrm{LmeB}_{\text {cF }}{ }^{24}$ | $\mathrm{HsaB}_{\mathrm{cc} 2} 2$ |  |  |  |  |  |  |  |
| 25 |  | $\mathrm{LmeB}_{\text {cF }} 25$ | $\mathrm{HsaB}_{\mathrm{cc} 2} 25$ |  |  |  |  |  | , |  |
| 26 |  | $\mathrm{LmeB}_{\text {cF }} 26$ | $\mathrm{HsaB}_{\mathrm{cc} 26}$ |  |  |  |  |  |  | - |
| 27 |  |  |  |  | DreBA $_{\text {FG }} 27$ |  | TniBA ${ }_{\text {cG }} 27$ |  |  |  |
| 28 |  |  | $\mathrm{HsaB}_{\mathrm{cc} 2} 28$ |  |  |  | TniBA ${ }_{\text {cG }} 28$ |  |  |  |
| 29 |  | $\mathrm{LmeB}_{\text {cF }} 29$ | $\mathrm{HsaB}_{\mathrm{cc} 2} 29$ |  |  |  |  |  |  |  |
| 30 |  |  | $\mathrm{HsaB}_{\mathrm{cc} 3} 30$ |  |  | OlaBA ${ }_{\text {cG }} 30$ |  |  |  |  |
| 31 |  | $\mathrm{LmeB}_{\text {cF }} 31$ | $\mathrm{HsaB}_{\mathrm{cc} 3} 31$ |  |  |  |  |  |  |  |
| 32 |  | $\mathrm{LmeB}_{\text {cr }} 32$ | $\mathrm{HsaB}_{\mathrm{cc} 3} 3$ |  |  |  |  |  |  |  |
| 33 |  | $\mathrm{LmeB}_{\mathrm{FG}} 33$ | $\mathrm{HsaB}_{\mathrm{cc} 3} 3$ |  |  |  |  |  |  |  |
| 34 |  | $\mathrm{LmeB}_{\text {cF }} 34$ | $\mathrm{HsaB}_{\mathrm{cc} 3} 3$ |  |  |  |  |  |  |  |
| 35 | XtrBcc 35 |  | $\mathrm{HsaB}_{\mathrm{cc} 3} 3$ |  |  |  |  |  | $)^{*}$ | - |
| 36 | $\mathrm{XtrB}_{\text {cc }} 36$ |  | $\mathrm{HsaB}_{\mathrm{cc}} 36$ |  |  |  |  |  |  |  |
| 37 | $\mathrm{XtrB}_{\mathrm{cc}} 37$ |  | $\mathrm{HsaB}_{\mathrm{cc}} 37$ |  |  |  |  |  |  |  |
| 38 | $\mathrm{XtrB}_{\mathrm{cc}} 38$ |  | $\mathrm{HsaB}_{\mathrm{cc} 3} 38$ |  |  |  |  |  |  |  |
| 39 |  |  |  |  |  | $\mathrm{OlaBA}_{\text {GH }} 39$ | $\mathrm{TniBA}_{\text {chi }} 39$ | H0, |  | - |
| 40 |  |  |  |  | DreBA ${ }_{\text {GH }} 40$ | OlaBA ${ }_{\text {GH }} 40$ |  |  |  |  |
| 42 |  |  |  | $\mathrm{PseB}_{\text {fH }} 42$ |  | $\mathrm{OlaBA}_{\text {GH }} 42$ |  |  |  |  |
| 43 |  |  | $\mathrm{HsaB}_{\mathrm{GH}} 43$ |  |  | OlaBA ${ }_{\text {GH }} 43$ |  |  |  | + |
| 44 |  |  | $\mathrm{HsaB}_{\mathrm{GH}} 44$ |  |  |  | $\mathrm{TniBA}_{\text {GH }} 44$ |  |  |  |
| 45 |  |  | $\mathrm{HsaB}_{\mathrm{GH}^{4} 4}$ |  | $\mathrm{DreBA}_{\text {G4 }} 45$ | $\mathrm{OlaBA}_{\text {GH }} 45$ | $\mathrm{TniBA}_{\text {GH }} 45$ |  | 为 | - |
| 46 |  |  | $\mathrm{HsaB}_{\mathrm{GH}^{4} 4}$ |  | $\mathrm{DreBA}_{\text {GH }} 46$ |  |  |  | Hex |  |
| 47 |  |  |  |  | DreBA $\mathrm{GH}_{\text {4 }} 47$ |  | $\mathrm{TniBA}_{\text {GH }} 47$ |  |  |  |
| 48 |  |  |  |  | $\mathrm{DreBA}_{\text {GH }} 48$ | $\mathrm{OlaBA}_{\text {GH }} 48$ |  |  |  |  |
| 49 |  |  | $\mathrm{HsaB}_{\mathrm{GH}} 49$ | $\mathrm{PseB}_{\text {fry }} 49$ |  |  |  |  |  |  |
| 50 | $\mathrm{XtrB}_{\mathrm{GH}} 5 \mathrm{O}_{\text {atcdef }}$ | $\mathrm{LmeB}_{\text {GH }} 50_{\text {atc }}$ | $\mathrm{HsaB}_{\text {GHi }} 50_{\text {abodef }}$ | $\mathrm{PseB}_{\mathrm{FH} 5} 5_{\text {atef }}$ | $\mathrm{DreBA}_{\text {GHi }} 50_{\text {bef }}$ |  |  |  |  |  |
| 51 | $\mathrm{XtrB}_{\mathrm{GH}} 51_{\mathrm{ab}}$ | $\mathrm{LmeB}_{\text {GH }} 55_{\text {alkd }}$ | $\mathrm{Hsab}_{\mathrm{GH}} 5 \mathrm{l}_{\text {abc }}$ | $\mathrm{PseB}_{\mathrm{FH} 5} 1_{\text {alad }}$ | $\mathrm{DreBA}_{\text {chi }} 51_{\text {bed }}$ |  |  |  |  |  |
| 52 | $\mathrm{XtrB}_{\text {ch }} 52_{\text {bade }}$ | $\mathrm{LmeB}_{\text {GH } 5} 52_{\text {abade }}$ | $\mathrm{HsaB}_{\text {GH }} 52_{\text {bade }}$ | $\mathrm{PseB}_{\text {FF }} 52_{\text {acd }}$ | $\mathrm{DreBA}_{\text {GH }} 52_{\text {acd }}$ |  |  |  |  |  |
| 53 |  |  |  |  |  | $\mathrm{OlaBA}_{\text {GH }} 53$ | $\mathrm{TniBA}_{\text {ch }} 53$ |  |  |  |
| 54 |  |  |  |  |  | OlaBA H $^{5} 5$ | $\mathrm{TniBA}_{45} 54$ |  |  |  |
| 55 |  |  |  |  | DreBA ${ }_{\text {HII }} 5$ |  | $\mathrm{TniBA}_{\mathrm{HI}} 55$ |  | H |  |
| 56 |  |  |  |  | DreBA ${ }_{\text {HII }} 56$ |  | $\mathrm{TniBA}_{H} 56$ |  |  |  |
| 57 |  |  | $\mathrm{HsaB}_{\mathrm{HI}} 57$ |  |  | $\mathrm{OlaBA}_{4,57}$ |  |  |  |  |
| 58 |  |  |  | $\mathrm{PseB}_{\mathrm{HI}} 58$ |  |  | $\mathrm{TniBA}_{\text {H5 }} 58$ |  |  |  |
| 59 |  |  |  | $\mathrm{PseB}_{\mathrm{HI}} 59$ |  |  |  | $\mathrm{DreBB}_{\mathrm{HI} 59}$ | \% | ( |
| 60 | $\mathrm{XtrB}_{\mathrm{GH}} 60_{\mathrm{a}}$ | $\mathrm{LmeB}_{\text {HII }} 60_{\text {ab }}$ | $\mathrm{HsaB}_{\mathrm{HI}} 60_{\mathrm{a}}$ | $\mathrm{PseB}_{\mathrm{Hf}} 60_{\mathrm{ab}}$ | $\mathrm{DreBA}_{\mathrm{HI}} 60_{\text {ab }}$ | $\mathrm{OlaBA}_{\mathrm{Hy} 60} \mathrm{ab}_{\text {ab }}$ |  |  | , |  |
| 61 |  |  |  | $\mathrm{PseB}_{\mathrm{HH}} 61$ |  |  |  | DreBB ${ }_{\text {H }} 61$ |  |  |
| 62 |  | $\mathrm{LmeB}_{\mathrm{HII}} 62_{\text {abc }}$ | $\mathrm{HsaB}_{\mathrm{Hf}} 62_{\text {abc }}$ | $\mathrm{PseB}_{\mathrm{Hf} 6} 62_{\text {abc }}$ | DreBA $\mathrm{Hf} 62_{\mathrm{b}}$ |  |  |  |  |  |
| 63 |  | $\mathrm{LmeB}_{\text {HII }} 63_{\text {ab }}$ | $\mathrm{HsaB}_{\mathrm{Hf}} 63_{\mathrm{ab}}$ | $\mathrm{PseB}_{\text {mil }} 63_{\text {ab }}$ | DreBA $\mathrm{HI}_{6} 3^{\text {b }}$ |  |  |  |  |  |
| 64 |  |  | $\mathrm{HsaB}_{\mathrm{J}} 64$ |  |  |  |  | DreBB ${ }_{\text {H1 } 64}$ |  |  |
| 65 | $\mathrm{XtrB}_{\mathrm{u}} 65_{\text {atc }}$ | $\mathrm{LmeB}_{16} 65_{\text {abod }}$ | $\mathrm{HsaB}_{\mathrm{H}} 65_{\text {abad }}$ |  | DreBA $\mathrm{L}_{6} 6 \mathrm{ababd}$ | $\mathrm{OlaBA}_{\mathrm{Hf} 6} 5_{\text {bc }}$ |  | $\mathrm{DreBB}_{\mathrm{Hf} 65_{\text {cd }}}$ |  |  |
| 66 |  | $L^{\text {LmeB] }} 66$ | $\mathrm{HsaB}_{\mathrm{J}} 66$ |  |  |  |  |  |  |  |
| 67 |  |  |  |  | DreBA ${ }^{4} 67$ |  | TniBA ${ }_{\text {HJ }} 67$ |  |  |  |
| 68 |  |  |  |  | DreBA ${ }_{4} 68$ |  | $\mathrm{TniBA}_{\text {H1 }} 68$ |  |  |  |
| 69 |  |  |  | 者 | VreBA ${ }_{4} 69$ |  |  |  |  |  |
| 70 | $\mathrm{XtrB}_{\mathrm{H}} 70_{\mathrm{ab}}$ | $\mathrm{LmeB}_{\mathrm{yj}} 70_{\text {ab }}$ | $\mathrm{HsaB}_{\mathrm{y}} 7 \mathrm{a}_{\text {ab }}$ |  |  | $\mathrm{OlaBA}_{\mathrm{Hf}} 70_{\mathrm{ab}}$ | $\mathrm{TniBA}_{\text {HJ }} 70_{\text {ab }}$ | $\mathrm{DreBB}_{\mathrm{H} 7} 7 \mathrm{O}_{\mathrm{b}}$ | $\mathrm{OlaBB}_{\mathrm{H}} 7 \mathrm{O}_{\mathrm{b}}$ | $\mathrm{TniBB}_{\mathrm{L}} 7 \mathrm{O}_{\mathrm{b}}$ |
| 71 |  | $\mathrm{LmeB}_{\text {IJ }} 71$ | $\mathrm{HsaB}_{\mathrm{L}} 71$ | - |  |  |  |  |  |  |
| 72 |  |  |  |  |  |  |  |  | $\mathrm{OlaBB}_{\text {JK }} 72$ | $\mathrm{TniBB}_{\text {JK }} 72$ |
| 73 |  |  |  |  |  |  |  |  | $\mathrm{OlaBB}_{\text {JK }} 73$ | $\mathrm{TniBE}_{\text {JK }} 73$ |
| 74 |  |  |  |  |  |  |  |  | $\mathrm{OlaBB}_{\text {JK }} 74$ | $\mathrm{TniBE}_{\text {IK }} 74$ |
| 75 |  |  |  |  |  | $\mathrm{OlaBA}_{\text {IK }} 75$ | $\mathrm{TniBA}_{\text {JK }} 75$ |  |  |  |
| 76 |  |  |  |  |  | $\mathrm{OlaBA}_{\text {IK }} 76$ | $\mathrm{TniBA}_{\text {JK }} 76$ |  |  |  |
| 77 |  |  |  |  | $\mathrm{DreBA}_{\text {JK }} 77$ |  | $\mathrm{TniBA}_{\text {JK }} 77$ |  |  |  |
| 78 |  |  |  | $\mathrm{PseB}_{1 \mathrm{IK}} 78$ |  |  |  |  | $\mathrm{OlaBB}_{\mathrm{JK}} 78$ |  |
| 79 | $\mathrm{XtrB}_{\text {J }} 79_{\mathrm{ab}}$ | $\mathrm{LmeB}_{\text {JK }} 79_{\text {atc }}$ | $\mathrm{HsaB}_{\text {JK }} 79_{\text {atc }}$ | $\mathrm{PseB}_{1 \mathrm{IK}} 79_{\text {alc }}$ | DreBA $_{\text {IK }} 79^{\text {be }}$ |  |  | $\mathrm{DreBB}_{\text {JK }} 79_{\text {bc }}$ | $\mathrm{OlaBB}_{\text {JK }} 79_{\text {abc }}$ | $\mathrm{TniBE}_{\text {JK }} 79_{\text {abc }}$ |
| 80 |  | $\mathrm{LmeB}_{\text {JK }} 80$ | $\mathrm{HsaB}_{\text {kL }} 80$ |  |  |  |  |  |  |  |
| 81 |  |  |  |  |  |  |  |  | $\mathrm{OlaBB}_{\text {KM }} 81$ | $\mathrm{TniBB}_{\text {KM }} 81$ |
| 82 |  |  |  |  |  |  |  |  | $\mathrm{OlaBB}_{\text {Kı }} 82$ | $\mathrm{TniBB}_{\text {Kı }} 82$ |


| 83 |  |  |  | $\mathrm{PseB}_{\text {kL }} 83$ | DreBA $_{\text {KL }} 83$ |  |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| 84 |  |  | $\mathrm{HsaB}_{\text {Kı }} 84$ | $\mathrm{PseB}_{\text {Kı }} 84$ |  |  |  |  |  |  |
| 85 |  |  |  | $\mathrm{PseB}_{\text {KL }} 85$ | DreBA ${ }_{\text {KL }} 85$ |  |  |  |  |  |
| 86 |  |  |  | $\mathrm{PseB}_{\text {KL }} 86$ | DreBA ${ }_{\text {KL }} 86$ |  |  |  |  |  |
| 87 | $\mathrm{XtrB}_{\mathrm{KL}} 87_{\text {badef }}$ | $\mathrm{LmeB}_{\text {KL }} .87_{\text {acdef }}$ | $\mathrm{HsaB}_{\mathrm{KL}} 87_{\text {abcdef }}$ | $\mathrm{PseB}_{\text {KL }} 87_{\text {abcedef }}$ | DreBA $_{\text {KL }} 87_{\text {abodef }}$ | OlaBA ${ }_{\text {Kı }} 87_{\text {badef }}$ | TniBA ${ }_{\text {KL }} 87_{\text {bedef }}$ | $\mathrm{DreBB}_{\text {ko }} 87_{\text {df }}$ | $\mathrm{OlaBB}_{\text {KM }} 87_{\text {de }}$ | $\mathrm{TniBB}_{\text {KM }} 87_{\text {de }}$ |
| 88 |  |  | $\mathrm{HsaB}_{\text {KL }} 88$ |  |  |  |  | $\mathrm{DreBB}_{\text {K0 }} 88$ |  |  |
| 89 |  |  | $\mathrm{HsaB}_{\text {KL }} 89$ | $\mathrm{PseB}_{\text {KL }} 89$ |  |  |  |  |  |  |
| 90 |  | $\mathrm{LmeB}_{\mathrm{KL}} 90_{\text {abad }}$ | $\mathrm{HsaB}_{\text {Kı }} 90_{\text {abcd }}$ | $\mathrm{PseB}_{\text {KL }} 900_{\text {alod }}$ | $\mathrm{DreBA}_{\mathrm{KLI}} 90_{\mathrm{b}}$ |  |  | $\mathrm{DreBB}_{\mathrm{K} 0} 90_{\text {abcd }}$ | $\mathrm{OlaBB}_{\text {KM }} 90_{\text {abc }}$ | $\mathrm{TniBB}_{\text {KM }} 90_{\text {bc }}$ |
| 91 |  |  |  | $\mathrm{PseB}_{\text {KL }} 91$ | DreBA ${ }_{\text {Kı }} 91$ | OlaBA ${ }_{\text {Kı }} 91$ | TniBA ${ }_{\text {Kı }} 91$ |  |  |  |
| 92 |  |  |  |  |  |  |  | $\mathrm{DreBB}_{\mathrm{K} 0} 92$ | $\mathrm{OlaBB}_{\text {KM }} 92$ |  |
| 93 |  |  |  |  |  |  |  | $\mathrm{DreBB}_{\text {K0 }} 93$ | $\mathrm{OlaBB}_{\text {м } 0} 93$ | $\mathrm{TniBB}_{\text {м0 }} 93$ |
| 94 |  |  |  | $\mathrm{PseB}_{\text {Kı }} 94$ | DreBA ${ }_{\text {KL }} 94$ |  |  |  |  |  |
| 95 |  |  |  | $\mathrm{PseB}_{\text {KL }} 95$ |  |  | TniBA $_{\text {Kı }} 95$ |  |  |  |
| 96 |  |  | $\mathrm{HsaB}_{\text {Kı }} 96$ |  |  | $\mathrm{OlaBA}_{\text {Kı }} 96$ |  |  |  |  |
| 98 |  |  | $\mathrm{HsaB}_{\mathrm{KL}} 98$ |  | DreBA ${ }_{\text {KL }} 98$ |  |  |  |  |  |
| 99 |  |  | $\mathrm{HSaB}_{\text {kL }} 99$ |  |  |  |  |  | $\mathrm{OlaBB}_{\text {KM }} 99$ |  |
| 100 |  | LmeB $^{\text {LL }} 100$ | $\mathrm{HsaB}_{\text {KL }} 100$ |  |  |  |  |  |  |  |
| 101 |  | $\mathrm{LmeB}_{\text {kL }} 101$ | $\mathrm{HsaB}_{\text {KL }} 101$ |  |  |  |  |  |  |  |
| 102 |  | $\mathrm{LmeB}_{\text {KL }} 102_{\text {ab }}$ | $\mathrm{HsaB}_{\mathrm{KL}} 102_{\mathrm{ab}}$ | $\mathrm{PseB}_{\text {Kı }} 102_{\mathrm{ab}}$ | $\mathrm{DreBA}_{\text {KL }} 102_{\text {ab }}$ | OlaBA ${ }_{\text {KL }} 102_{\text {b }}$ | TniBA ${ }_{\text {KL }} 102_{\mathrm{b}}$ |  |  |  |
| 103 |  |  |  |  | DreBA $_{\text {KL }} 103$ | OlaBA ${ }_{\text {KL }} 103$ |  |  |  |  |
| 104 |  |  | $\mathrm{HsaB}_{\text {kL }} 104_{\text {bcde }}$ | $\mathrm{PseB}_{\text {KL }} 104_{\text {abcde }}$ | DreBA ${ }_{\text {KL }} 104_{\text {abcde }}$ | OlaBA ${ }_{\text {KL }} 104_{\text {abcd }}$ | TniBA ${ }_{\text {KL }} 104_{\text {abcd }}$ | DreBB ${ }_{\text {Ko }} 104_{\text {abee }}$ |  | TniBB ${ }_{\text {Mo }} 104_{\text {b }}$ |
| 105 |  |  |  | $\mathrm{PseB}_{\text {kL }} 105_{\text {abc }}$ | DreBA ${ }_{\text {KL }} 105_{\text {abc }}$ | OlaBA ${ }_{\text {KL }} 105_{\text {ac }}$ | TniBA ${ }_{\text {KL }} 105_{\text {bc }}$ |  |  |  |
| 106 |  |  |  | $\mathrm{PseB}_{\text {KL }} 106$ | $\operatorname{DreBA}_{\text {KL }} 106$ |  |  |  |  |  |
| 107 |  |  | $\mathrm{HsaB}_{\text {KL }} 107$ |  | DreBA $_{\text {KL }} 107$ |  |  |  |  |  |
| 108 |  | $\mathrm{LmeB}_{\text {KL }} 108$ | $\mathrm{HsaB}_{\text {KL }} 108$ | $\mathrm{PseB}_{\text {KL }} 108$ | DreBA ${ }_{\text {KL }} 108$ |  |  |  |  |  |
| 109 |  |  |  |  |  | OlaBA ${ }_{\text {LM }} 109$ | TniBA ${ }_{\text {LM }} 109$ |  |  |  |
| 110 | $\mathrm{XtrB}_{\text {KL }} 110_{\text {abc }}$ | $\mathrm{LmeB}_{\text {LM }} 110_{\text {abc }}$ | $\mathrm{HsaB}_{\text {LM }} 110_{\mathrm{b}}$ |  | DreBA ${ }_{\text {LM }} 10_{\text {abc }}$ |  | TniBA ${ }_{\text {KL }} 110_{\text {bc }}$ |  |  |  |
| 111 |  | $\mathrm{LmeB}_{\text {LM }} 111$ | $\mathrm{HsaB}_{\text {LM }} 111$ | 隹mmmmm |  |  |  |  |  |  |
| 112 |  | $\mathrm{LmeB}_{\text {LM }} 112$ | $\mathrm{HsaB}_{\mathrm{LM}} 112$ | 考mmmmmm |  |  |  |  |  |  |
| 113 | $\mathrm{XtrB}_{\text {KL }} 113_{\text {ab }}$ | $\mathrm{LmeB}_{\text {LM }} 113_{\text {ab }}$ | $\mathrm{HsaB}_{\text {LM }} 113_{\text {ab }}$ | － |  | $\mathrm{OlaBA}_{\text {LM }} 113_{\text {bc }}$ | $\mathrm{TniBA}_{\text {LM }} 113_{\text {bc }}$ |  |  |  |
| 114 | $\mathrm{XtrB}_{\text {KL }} 114_{\mathrm{b}}$ | $\mathrm{LmeB}_{\text {LM }} 114_{\text {ab }}$ | $\mathrm{HsaB}_{\text {LM }} 114_{\text {ab }}$ |  |  |  |  |  |  |  |
| 115 |  |  | $\mathrm{HsaB}_{\text {LM }} 115$ |  |  |  |  | $\mathrm{DreBB}_{\text {Ko }} 115$ |  |  |
| 116 |  | LmeB $^{\text {LM }} 116$ | $\mathrm{HsaB}_{\text {LM }} 116$ |  |  |  |  |  |  |  |
| 117 |  |  | $\mathrm{HsaB}_{\text {LM }} 117$ |  |  |  |  |  | $\mathrm{OlaBB}_{\text {KM }} 117$ |  |
| 118 | $\mathrm{XtrB}_{\mathrm{LM}} 118_{\text {abcde }}$ | $\mathrm{LmeB}_{\text {LM }} 118_{\text {abcde }}$ | $\mathrm{HsaB}_{\mathrm{LM}} 118_{\text {abcde }}$ |  |  | $\mathrm{OlaBA}_{\text {LM }} 118_{\mathrm{b}}$ | $\mathrm{TniBA}_{\mathrm{LM}} 118_{\text {bc }}$ |  |  |  |
| 119 |  |  |  | V | DreBA ${ }_{\text {LM }} 119$ | OlaBA ${ }_{\text {LM }} 119$ |  |  |  |  |
| 120 |  |  |  | VmmmIMIM／ | － $\mathrm{DreBA}_{\text {LM }} 120$ | $\mathrm{OlaBA}_{\text {LM }} 120$ |  |  |  |  |
| 121 |  |  |  |  |  | OlaBA ${ }_{\text {LM }} 121$ | TniBA ${ }_{\text {LM }} 121$ |  |  |  |
| 122 |  |  |  |  |  | $\mathrm{OlaBA}_{\text {LM }} 122$ | $\mathrm{TniBA}_{\text {LM }} 122$ |  |  |  |
| 123 |  |  |  |  |  | OlaBA ${ }_{\text {LM }} 123$ | $\mathrm{TniBA}_{\text {LM }} 123$ |  |  |  |
| 124 |  |  |  | 者 |  | $\mathrm{OlaBA}_{\text {LM }} 124$ | $\mathrm{TniBA}_{\text {LM }} 124$ |  |  |  |
| 125 |  |  |  |  |  | $\mathrm{OlaBA}_{\text {LM }} 125$ | $\mathrm{TniBA}_{\text {LM }} 125$ |  |  |  |
| 126 |  |  |  |  |  | $\mathrm{OlaBA}_{\text {LM }} 126$ | $\mathrm{TniBA}_{\text {LM }} 126$ |  |  |  |
| 127 |  |  |  |  | 俍 $\mathrm{DreBA}_{\text {LM }} 127$ | OlaBA ${ }_{\text {LM }} 127$ | TniBA ${ }_{\text {LM }} 127$ |  |  |  |
| 128 |  |  | $\mathrm{HsaB}_{\text {LM }} 128$ |  |  | OlaBA ${ }_{\text {LM }} 128$ |  |  |  |  |
| 129 |  |  | $\mathrm{HsaB}_{\text {LM }} 129$ | 年mmmmmm |  |  |  |  |  |  |
| 130 |  | $\mathrm{LmeB}_{\text {LM }} 130$ | $\mathrm{HsaB}_{\text {LM }} 130$ | 寺 |  |  |  |  |  |  |
| 131 |  |  | $\mathrm{HsaB}_{\text {LM }} 131$ |  |  |  |  |  |  | $\mathrm{TniBB}_{\text {км }} 131$ |
| 132 |  |  | $\mathrm{HsaB}_{\text {LM }} 132$ |  | ＜${\text { DreBA }{ }_{\text {LM }} 132}^{\text {a }}$ |  |  |  |  |  |
| 133 |  | $\mathrm{LmeB}_{\text {LM }} 133$ | $\mathrm{HsaB}_{\text {LM }} 133$ | － |  |  |  |  |  |  |
| 134 |  | $\mathrm{LmeB}_{\text {LM }} 133_{\text {acd }}$ | $\mathrm{Hsab}_{\mathrm{LM}} 133_{\text {abcd }}$ |  | \}  DreBA  _ {  LM  } 1 3 4 _ {  abod  } |  | $\mathrm{TniBA}_{\text {LM }} 134_{\text {bc }}$ |  |  |  |
| 135 |  | $\mathrm{LmeB}_{\text {LM }} 135$ | $\mathrm{HsaB}_{\text {LM }} 135$ |  |  | OlabA ${ }_{\text {LM }} 135$ |  |  |  |  |
| 136 |  |  |  |  |  | OlaBA ${ }_{\text {MN }} 136$ | TniBA ${ }_{\text {MN }} 136$ |  |  |  |
| 137 |  |  |  |  |  | $\mathrm{OlaBA}_{\text {M1 }} 137$ | TniBA ${ }_{\text {MN }} 137$ |  |  |  |
| 138 |  |  |  | VMm） |  | OlaBA ${ }_{\text {MN }} 138$ | TniBA ${ }_{\text {MN }} 138$ |  |  |  |
| 139 | $\mathrm{XtrB}_{\text {MN }} 139_{\mathrm{bc}}$ | $\mathrm{LmeB}_{\text {MN }} 139_{\text {bcd }}$ | $\mathrm{HsaB}_{\text {MN }} 139_{\text {abcd }}$ |  | DreBA ${ }_{\text {MN }} 139_{\text {brde }}$ | OlaBA MN $^{13} 139_{\text {acde }}$ | $\mathrm{TniBA}_{\text {MN }} 139$ acde |  |  |  |
| 140 | $\mathrm{XtrB}_{\text {MN }} 140_{\mathrm{b}}$ | $\mathrm{LmeB}_{\text {MN }} 140{ }_{\text {bc }}$ | $\mathrm{HsaB}_{\text {MN }} 140_{\text {bc }}$ |  | DreBA ${ }_{\text {MN }} 140_{\text {abc }}$ | OlaBA ${ }_{\text {MN }} 140{ }_{\text {abc }}$ | $\mathrm{TniBA}_{\text {MN }} 140_{\text {abc }}$ |  |  |  |
| 141 |  | $\mathrm{LmeB}_{\text {MN }} 141$ | $\mathrm{HsaB}_{\text {MN }} 141$ |  | DreBA ${ }_{\text {MN }} 141$ | $\mathrm{OlaBA}_{\text {MN }} 141$ | TniBA ${ }_{\text {MN }} 141$ |  |  |  |
| 142 |  |  |  |  |  | $\mathrm{OlaBA}_{\text {MN }} 142$ | TniBA ${ }_{\text {M }} 142$ |  |  |  |
| 143 |  |  |  |  |  | $\mathrm{OlaBA}_{\text {MN }} 143$ | TniBA ${ }_{\text {M }} 143$ |  |  |  |
| 144 |  |  |  |  | DreBA ${ }_{\text {MN }} 144$ |  | TniBA ${ }_{\text {MN }} 144$ |  |  |  |
| 145 |  |  |  |  | DreBA ${ }_{\text {MN }} 145$ |  | $\mathrm{TniBA}_{\text {M }} 145$ |  |  |  |
| 146 |  |  |  |  |  |  | TniBA ${ }_{\text {MN }} 146$ |  |  |  |
| 147 |  |  |  | 多mmmmmmm | －${ }_{\text {DreBA }}^{\text {N0 }} 147$ | OlaBA ${ }_{\text {No }} 147$ |  |  |  |  |
| 148 |  |  |  |  |  | OlaBA ${ }_{\text {NO }} 148$ |  |  |  |  |
| 149 |  |  |  |  | － $\mathrm{DreBA}_{\text {NO }} 149$ | $\mathrm{OlaBA}_{\text {No }} 149$ |  |  |  |  |
| 150 |  | $\mathrm{LmeB}_{\text {MN }} 150$ | $\mathrm{HsaB}_{\text {Ms }} 150$ |  |  |  |  |  |  |  |
| 151 |  | $\mathrm{LmeB}_{\text {MN }} 151$ | $\mathrm{HsaB}_{\text {MN }} 151$ |  |  |  |  |  |  |  |
| 152 |  | $\mathrm{LmeB}_{\text {MN }} 152$ | $\mathrm{HsaB}_{\text {MN }} 152$ | － |  |  |  |  |  |  |
| 153 |  | $\mathrm{LmeB}_{\text {MN }} 153$ | $\mathrm{HsaB}_{\text {MN }} 153$ |  |  |  |  |  |  |  |
| 154 |  | $\mathrm{LmeB}_{\mathrm{No}} 154_{\mathrm{b}}$ | $\mathrm{HsaB}_{\mathrm{NO} 1} 154_{\text {abc }}$ | Vmomm |  | OlaBA ${ }_{\text {No }} 154_{\text {ab }}$ | $\mathrm{TniBA}_{\text {No }} 154_{\text {ab }}$ |  |  |  |
| 155 |  |  |  |  | DreBA ${ }_{\text {No }} 155$ | OlaBA NO 155 |  |  |  |  |
| 156 |  |  |  |  |  | $\mathrm{OlaBA}_{\text {No }} 156$ | $\mathrm{TniBA}_{\text {N0 }} 156$ |  |  |  |
| 157 |  |  |  |  | DreBA ${ }_{\text {NO }} 157$ |  | $\mathrm{TniBA}_{\text {No }} 157$ |  |  |  |
| 158 |  |  |  |  | － $\mathrm{DreBA}_{\text {N0 }} 158$ |  | $\mathrm{TniBA}_{\text {No }} 158$ |  |  |  |
| 159 |  |  | $\mathrm{HsaB}_{\text {vo }} 159$ |  |  |  |  |  | $\mathrm{OlaBB}_{\text {M0 }} 159$ |  |
| 160 |  |  | $\mathrm{HsaB}_{\text {No }} 160$ |  |  | OlaBA ${ }_{\text {No }} 160$ |  |  |  |  |
| 161 | $\mathrm{XtrB}_{\text {No }} 161_{\text {ab }}$ | $\mathrm{LmeB}_{\text {No }} 161_{\mathrm{a}}$ | $\mathrm{HsaB}_{\text {No }} 161_{\text {abc }}$ |  | DreBA ${ }_{\text {No }} 161_{\text {abc }}$ | $\mathrm{OlaBA}_{\mathrm{NO}^{161}} 161_{\text {abc }}$ | TniB $^{\text {N0 }} 1611_{\text {ab }}$ | DreBB ${ }_{\text {K0 }} 161_{\text {bc }}$ | $\mathrm{OlaBB}_{\text {м0 }} 161_{\text {bc }}$ | $\mathrm{TniBB}_{\text {M0 }} 161_{\text {bc }}$ |




| Homo sapiens human Hox B |  |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: |
| PFC | Dist to 3 ' gene | Length Sequence |  |  |  |
| $\mathrm{HsaB}_{\mathrm{cg}} 22$ | 96,213 | 23 CCACtGgGaggictaancctita |  |  |  |
| $\mathrm{HsaB}_{\mathrm{cG}} 23$ | 95,483 | 28 tatttatacaatanaittttttanaan |  |  |  |
|  |  | TAAAGGGGAGATCTAAGCAAATGATATGAACGGTCAGGTCAAG aAttGgCCACACAATTGAACGAAGTACATTTTTAGCATGTTGGT agatatcccattgaanatgcttgccatggangcatggGattc |  |  |  |
| $\mathrm{HsaB}_{\mathrm{cG}} 24$ | 84,917 | 159 AGGAAACTGGTTAGAGATCCCCCTACGGA |  |  |  |
| $\mathrm{HsaB}_{\mathrm{cg}} 25$ | 80,145 | 36 ttgatticclaagigcaggiaccettcgcagcctga |  |  |  |
| $\mathrm{HsaB}_{\mathrm{c}} 26$ | 78,392 | 21 gagctegttgactttatata |  |  |  |
| $\mathrm{HsaB}_{\mathrm{cG}} 28$ | 51,295 | 31 Cataiagaacttictagticatccttggicc |  |  |  |
| $\mathrm{HsaB}_{\mathrm{cG}} 29$ | 46,437 | 29 gaaatttttaanagcatctttigctgct |  |  |  |
| $\mathrm{HsaB}_{\mathrm{c}} 30$ | 44,661 | 21 cttattgatgagttgataia |  |  |  |
| $\mathrm{HsaB}_{\mathrm{cG}} 31$ | 43,671 | 16 Cactatgcctgicc |  |  |  |
| $\mathrm{HsaB}_{\mathrm{cG}} 32$ | 14,496 | 29 GTCAGTTTACGTAGGTCTTCACCCTTTCT <br> itaanttrantitanaanaacaggatacatticagtantccatt aAACATCAGGGGTGAATTGTAACAGAATCCAATTCCATATTTGA |  |  |  |
| $\mathrm{HsaB}_{\mathrm{cG}} 33$ | 30,201 | 89 T |  |  |  |
| $\mathrm{HsaB}_{\text {cG }} 34$ | 14,496 | 29 Gtcagittacgragatcttcaccetttct |  |  |  |
| $\mathrm{HsaB}_{\mathrm{cG}} 35$ | 11,355 | 23 GGccctggacgittctaaggigi | HsaBkı99 | 7,273 | 23 CGcticctctagaccagaggcaga |
| $\mathrm{HsaB}_{\mathrm{cG}} 36$ | 3,545 | 22 gtanatttcagctaagcttatt |  |  | ctgtaicatcccgaagctgccagtagagtccgicttagaccaa |
| $\mathrm{HsaB}_{\mathrm{cG}} 37$ | 1,319 | 24 agtacaggaggictaaaactcag | HsaBkı $10{ }^{*}$ | 7,248 | 51 gttcacag atgGaanatcanaicagggacgiggccgactanctcctatccg |
|  |  | cCgattgatttatgtcgaagctgacgettratcaggcagtcgaa AAAACTTTGACCAATCATTTTGCAAGGAGAGCTGAGACGGGCT | HsaBkı101 | 4,511 | 55 antggiactitt |
| $\mathrm{HsaB}_{\mathrm{cG}} 38^{*}$ | 264 | 118 getccactatacttrgttgcctaagaagttg |  |  | AAGTAATGACCTTCGCAAAATTCAATATGACCCAGGGAACTGC |
| $\mathrm{HsaB}_{\text {GH }} 43$ | 8,164 | 15 тсттссссатстс |  |  |  |
| $\mathrm{HsaB}_{\text {GH }} 44$ | 5,323 | 19 tcattaggaatgcaaatig | $\mathrm{HsaB}_{k} 102{ }^{*}$ | 4.357 | GTTTGAGAGCCTCGTGACGCGCCTAGCTGGTTAACAAAGACTGC 200 caAAGTATGAGATTAACACGAAAACT |
|  |  | gCantanatatatgaccgctatanaigttatagcgiataa |  | 4,357 | taccctgragatccganttgigtangaitttgtigtcacaa |
| $\mathrm{HsaB}_{\mathrm{GH}} 45^{*}$ | 4,945 | 84 ATtTCTGAAGGTTAAGAAACTAAACAGCAGCAAAGCAAACA | $\mathrm{HsaB}_{\text {kL }} 104{ }_{\text {bcde }}$ | 1,606 | 69 attcgtatctagggaatatgtagt |
| $\mathrm{HsaB}_{\text {GH }} 46$ | 4,502 | 38 AGACGTCTGGGGGCGGGCAGCCTCTTGAtTCTtTtaca | HsaBkı 107 | 1,472 | 34 tttctanatgagaicagtctgataictgiccaca |
| $\mathrm{HsaB}_{\text {GH }} 49$ | 2,390 | 35 TTTGTAAAGCCTCCACTTTTGTTGCTCAAAATTTT | HsaBkı ${ }^{108}{ }^{*}$ | 59 | 58 Aattcancicagagatteg |
|  |  |  | $\mathrm{HsaB}_{\text {LM }}{ }^{110_{b}{ }^{*}}$ | 23,888 | 13 catctttaatcac |
|  |  | ACAGCCGTTTTTTGGGGGGCCGGTTATCCAGCCAATTCCCTCCA | $\mathrm{HsaB}_{\text {LM }} 111$ | 23,080 | 26 TgTAcagagtgacaitaganatanat |
|  |  | CacgatagtanacctitatcantantahgGanattgatcatta |  |  |  |
|  |  |  | $\mathrm{HsaB}_{\mathrm{Lm}} 112$ | 22,942 | 90 TtATCTCAGAGGACTCCAAATTGTGGTGGCTGGTTTGTTTTCCTT |
| $\mathrm{HsaB}_{\text {GH }} 5 \mathrm{O}_{\text {abcset }}{ }^{*}$ | 1,227 | 342 AGAGGAAGCTGTATGATTTGATAACTTGTATTA |  |  |  |
| $\mathrm{HsaB}_{\mathrm{GH} 5} 5 \mathrm{labc}_{\text {ach }}$ | 748 | 71 AACAGATTTAATCCGTATTCATTCTC |  |  | acagatgittctaggigittgcaggitticagagiattitatatt acaangangctagccagtcceatagctcanactctaacangca aAtagataatcangangacanatg ccctctittgigaigcctig |
|  |  | CCAataggatgcggagtctctaatggatccanatgatcatgai | $\mathrm{HsaB}_{\mathrm{LM}}{ }^{1133_{\text {ab }}{ }^{*}}$ | 22,517 | 156 CTCCAGTATTAATTTTCATTTTC |
|  |  | aAGACAGTGCAAAATATGAAACAACTCATtTGCAGGGAAGTAA atcaccGaianctgittatganctggcatcccttcticgaantg | $\mathrm{HsaB}_{\mathrm{Lc}} 114_{\text {aba }}{ }^{*}$ | 21,660 | aAagttcacagccattctgtciagacaagagctaagaaaattg 72 tgagaattatacagaanaccattaatcac |
| $\mathrm{HsaB}_{\text {Gr }} 52_{\text {bccoe }}{ }^{*}$ | 480 | 153 TAAAGGGAGGACCTCTTTAAGTG | Hsabim ${ }^{115}$ | 21,308 | 20 cacaactganagaigaaca |
| $\mathrm{HsaB}_{\text {H57* }}{ }^{\text { }}$ | 1,889 | 34 attictoctaagttctclcancaicatganactg |  |  |  |
| $\mathrm{HsaB}_{41} 6 \mathrm{O}_{\mathrm{a}}$ | 1,868 | 17 CAACATGAAACTGCCTA | $\mathrm{HsaB}_{\mathrm{cm}} 116$ |  | CACGTTTTCTAGGCGATTAGCTCAGTATTAGAACCACAAAATGA 79 CCTCGGAAGCCCCTTGACCTCGCTGAGACTGGACA |
|  |  | agcatgcgcgetgiggccaattgitacangigitcttaggitta CTGTGAAGAGAATGTATTCTGTATCCGTGAATTGCTTTATGGGGG | $\mathrm{HsaB}_{\mathrm{LM}} 117$ | 19,434 | 26 TTGGTTTGCATtTTGTAAATTGTTTC |
|  |  | GGAGGGAGGGCTAATTATATATtTTGGTTGTTCCTCTATACTTTGG |  |  |  |
|  |  | TCTGTTGTCTGCGCCTGAAAAGGGCGGAAGAGTTACAATAAAGT tTACAAGCGAGAACCCGAGACTGGCCCGGCCAGCGCTCCTCAT |  |  | GGTCATAGTGGTCCTTTGAGCCTGAAATCGAAAGACCCCCTTCC cсCtcccagtcacgtaattcattaAataattantcccgaggttg |
| HsaB ${ }_{\text {H1 }} 62_{\text {abc }}$ | 1,623 | 223 тT |  |  | саGAATAGATAGGTATtCGTCAGGAAAATCGCAGGCTCGGTCTC |
|  |  |  |  |  | CCTGTTTCTGACGTGCAATTCAACTGTCCTTTGAAAAACAAGCC |
|  |  | ATTATTGATCATATTTTATAAATCCAACGCCACACAATTTTTTCC | $\mathrm{HsaB}_{\text {LM }} 118 \mathrm{abaccos}^{*}$ | 16,792 | 393 AgAGAGAGAGAGAGAAAGAAAGGGAGACAGCAATAAA |
|  |  |  | $\mathrm{HsaB}_{\mathrm{Lm}} 128$ | 12,557 | 22 тестесСтСАААСтСССаGсст |
|  |  | AGGGGGTCCTTTTTGGGGTAAATCTGGACTCTAATTCTGTAATAT ATCAAGGATCTCGTAAACCGACACTAAAACGTCCCTCCTA | $\mathrm{HsaB}_{\mathrm{Lm}} 129$ | 10,193 | 27 taittaatteatttatcatanatca |
|  |  | 238 CAAAATCATCCGGGCCAAATT |  |  | tttiatatttgaggcantcgaangictttcgctgattggtca |
| $\mathrm{HsaB}_{\text {H6 }} 63_{\text {ab }}$ | 238 | 238 CaAATCATCCGGCCAAATT |  |  |  |
| $\mathrm{HsaB}_{10} 64$ | 9,547 | 23 ttaattaanggagttanaac | $\mathrm{HsaB}_{\mathrm{Lm}} 130$ | 7,873 | 108 agaancatccagtctatca |
|  |  |  | $\mathrm{HsaB}_{\mathrm{cm}} 131$ | 7,665 | 20 tgcagcetcecttcttctic |
|  |  | AGATGGCGACTGAGAAAAGGGTTGCTGGTGGAGCAGCCATGAA | $\mathrm{HsaB}_{\text {LM }} 132^{*}$ | 4,597 |  |
|  |  | GTGATAGAAGTTTATGAGTGGTTGAATCCAGCGATTGGCCGGCG CCGGTCATGTGGCCGGGCGATCGTGAACATGAACTTTTTATCAT | $\mathrm{HsaB}_{\mathrm{Lm}} 133$ | 3,473 | cagaggccattittacgaagagtaggGcgtaccagtcttatagg 51 GCAACCA |
| $\mathrm{HsaB}_{\text {J }} 65_{\text {abca }}{ }^{*}$ | 7,032 | 209 TTCCCTGGTGGTTATAATGCAGCATTCTTTTGG |  |  | Ctcgtcgatggctanggraagctggactganataggctatcagt |
| $\mathrm{HsaB}_{1 / 66}$ | 5,717 | 15 attittganattana |  |  | TTGTGAATGGCGGAGAGTATGTCTCAATGATTTATGGCCCATAT |
| $\mathrm{HsaB}_{\\|} 7 \mathrm{O}_{\text {ab }}{ }^{*}$ | 103 | ccaatctcgaatatactactantaggcgeggcgctcga 61 AAACACAACAAATCATA | $\mathrm{HsaB}_{\mathrm{Lm}} 134_{\text {abcad }}{ }^{*}$ | 3,072 | 133 C |
|  |  |  | $\mathrm{HsaB}_{\mathrm{L}}{ }^{135}{ }^{\text {* }}$ | 248 | cctattGatgtcagitcccttticagttctaigat |
|  |  | ACTACCTAGAAGTAAGAAGAGGAGCCTCAGAAGAAAACAAAG 87 TTCTATTTTATTAATTTTCTATGTGTTGTGTTTGTAGTCTTGTCT |  |  |  |
| $\mathrm{HsaB}_{1 /} 71$ | 2,259 |  | $\mathrm{HsaB}_{\text {mp }} 139_{\text {abco }}{ }^{*}$ | 4,581 | 38 Tcttgacttancgitanaacaggitatattigacaia |
|  |  | cctaacgattctcgaatcgicattattigtaaccatagagcatg |  |  |  |
|  |  | Aattacctcttcag gicatcagcgacaattacgactggtcaic |  |  | AGAGATGTCTGGGCCTGCAGAAGTCCAGCATTGCTCAAAAAAG |
|  |  |  |  |  | cgtgtitictagtgatcattitcatatatattattgattatagc |
|  |  | CCCCCCCCATATTTGGCCGCATACATAGCAAAACGAAGTACAG |  |  | CTGTTAAATATтTTCTTTTTTGTATTATTTATCCCCCTACATTAT |
|  |  | TGGCATCGCTATAATTCATTAATACATCATAAATCGTGAAGCACA |  |  |  |
| $\mathrm{Hsab}_{\mathrm{Jk}} 79^{\text {abc }}$ | 267 | GCATGTTTAATGTGAACTCTCCCCTCCCCATCTGTGTTCTAACTT | ${ }_{\text {HsaB }}^{\text {mV }}$ (40 ${ }_{\text {bc }}$ | 4,478 |  |
| $\mathrm{HsaB}_{k 1} 80$ | 13,558 | 53 attiatah | Hsabmı 141 | 3,994 | tcacgitacccgangcceanccaccattggatctanaatgan |
| $\mathrm{HsaB}_{\text {kI }} 84$ | 13,483 | 19 gittcagctgaaactict | $\mathrm{HsaB}_{\text {mv }} 150$ | 3,495 | 52 ACAAAGAAA ${ }^{\text {a }}$ |
|  |  |  | $\mathrm{HsaB}_{\text {Mn }} 151$ | 3,013 | 33 Aattgrangcgatgiccccgcattccttantta |
|  |  | AAAAGTCGCTATGACATTTAGATGTCAAATGGATAGGGGTTTTA |  |  | TATAATTTAAAGGCATAAGAGGGTGCAAAGTTTGATTGGGATCA |
|  |  | ACCCCTCACTGGCTCGAGAAAGTCACGTGAGGTCCATAAAGTT agtittatggttrthggagagttgacaccgcgcagtatatticaca | $\mathrm{HsaB}_{\text {MN }} 152^{*}$ | 2,732 | 107 АTTAATAACATTTTCTG |
| $\mathrm{HsaB}_{\mathrm{kL}} 88_{\text {abocoef }}{ }^{*}$ | 12,115 | 204 тTCTCCAGAATGTTAAGTGACACTtTAAC |  |  | CTCTGGTGTTTTTGAATCAATTAAACCAAATAATGCTCTCTGTTT tCCACCAGGCCCAGACGAGCGATTGGCGGAGGCCGGTCCCGTG |
| $\mathrm{HsaB}_{\mathrm{kL}} 88$ | 12,418 | 40 CCAACtGatcttccctettgattaggagttaggitcctit |  |  | aCCACGAATTCCCTGTAATTTCGCTGGAGTCCTGGGTTTAATAG |
| $\mathrm{HsaB}_{\mathrm{kl}} 89$ | 11,577 | 35 GGGAAAAGGGTtTGTGTGGGGGATCCATGCtccct | $\mathrm{HsaB}_{\text {MN }} 153^{*}$ | 328 | 180 AAAG |
| $\mathrm{HsaB}_{\mathrm{kL}} 96$ | 9,043 | 18 Cattrttgaagitttica |  |  | tatttattanaittctttaitantagganaggGganagtatt |
|  |  |  | $\mathrm{HsaB}_{\text {no }} 154_{\text {asc }}{ }^{*}$ | 11,844 | 73 attgtacattatttcatagattaantai |
| $\mathrm{HsaB}_{\mathrm{kL}} 90_{\text {abod }}$ | 8,297 | 84 tCatcantanttcatagagtccgagatcatccagaggtca <br> ittgcatcctaanagGgaanatcancgctcatatctcatcanta 84 ATtCATAGAGTCCGGGATCATGCAGAGGTCAGCAGACGGG | $\mathrm{HsaB}_{\text {no }} 159$ | 5,635 | 20 ccttcaggttagataatat |
|  |  |  | $\mathrm{HsaB}_{\text {No }} 160$ | 2,483 | 25 ССтССтСССАТСТСААТттТССтTT |
|  |  |  |  |  |  |
| HsaBkı ${ }^{\text {8** }}$ | 8,288 |  | $\mathrm{HsaB}_{\text {no }} 161_{\text {abo }}{ }^{\text {* }}$ | 289 | 64 AATGCTTGGGGGGTGATGGA |


| Polypterus senegalus bichir Hox B |  |  |
| :---: | :---: | :---: |
| PFC | Dist to 3' gene | Length Sequence |
| PseB ${ }_{\text {AC }}{ }^{2}$ | 16,089 | 26 AaACAACGTCACCTTTCTGGAGAAAT |
| PseB $\mathrm{AC}^{\text {3 }}$ | 5,963 | 28 afttaattagtcantacgattagtgct |
| PseB ${ }_{\text {AC }} 4$ | 3,355 | 22 tttantgicacgitgantacat |
| PseB ${ }_{\text {cF }} 16$ | -107 | 14 TTTtTGCttitcit |
| PseB ${ }_{\text {cF }} 17$ | -1,106 | 27 AaATCAGTGTtTAACCTTGACCTTGAA |
| PseB ${ }_{\text {cF }} 18$ | 7,372 | 27 tttgitacgitactgcgattcatcagt |
| PseB ${ }_{\text {cF }} 19$ | 336 | 24 ttgaahtggtcantgtatacatt |
| PseB $\mathrm{cF}^{2} 20$ | 53 | tTGAAAAGTCAATTGAGAAGAACCCTTTGCCTTCGTTAAAATATGT 53 CCCAGCA |
| PseB ${ }_{\text {FH }} 21$ | in 10 | 16 tgatctgattcatatt |
| PseB ${ }_{\text {Fr }} 42$ | 2,782 | 26 atganacctgtattigagantgcaga |
| PseB ${ }_{\text {FH }} 49$ | 2,104 | 34 TTTGTAATGTTTCATTAAGTTTCCGCAGAATTTT |
|  |  | CTCGTAAAAATTTAACAGCTTGTGTTCCTTTGCATTCCTGTATAAAC AACGCGCAACCTTGCCGTTTATGGGATTTGTTAAACCATTTACCGC TTTATTTTTCTATTACGGCAGATACAATATCCACGGGTATACAACTT aAATATACATGAAACACATATTTATAACAAATCATTAAAACGTTA aAtatgaccacctcguttcctitatatanacgtttactggtggaata |
| $\mathrm{PseB}_{\text {FH }} 50_{\text {abef }}$ | 986 | 342 ctgcgitttcatangatgttcta |
|  |  | tgatttgatgcaangcgacagatatctcattanctancccgattga |
| PseB $\mathrm{BrH} 51_{\text {abca }}$ | 528 | 74 acatattantgcgeatattctccectce |
|  |  | gTCCGTGTGGAAAACCTGAAGCCAATGGGATGGAGATCCGGTAAT agatcianattatcatcaanagactctacanaitatganacaict CATTTGCGGCGGAGTAAATCACAGAAAACTGTTTATGAACTGGCA |
| PseB ${ }_{\text {FH }} 52_{\text {acd }}$ | 352 | 163 Cccettcttgganatgcantgcgagaic |
| PseB ${ }_{\text {H15 }} 5$ | 1,944 | 27 TTGGTGTGTGAAGAAACAAAGAACTGA |
| PseB ${ }_{\text {H15 }} 5$ | 1,815 | 36 AaAtGTGAAACTTTTTATtAGAATGGTtTCTTAATT |
| PseB $\mathrm{Hl}_{16} 6 \mathrm{ab}_{\text {ab }}$ | 1,680 | 24 Cancatganactgcctattiatge |
| PseB $\mathrm{HH}_{61}$ | 1,624 | 16 anatcangatatttit |
|  |  | agcatgcgcacagitanaaaanatgtancgtgaccctctgittact gTGAAAAGAATGTATACTGTACTCGTGAATTACTTTATGGGGACTC CGTGATATACTCTGTTGTTTTGCGCTACTTTGTTCTATTGTCTAAACC TGGAAGCAGCGGAAGAAAGGGCACAATAAGTTTACAAGCGAG |
| PseB $\mathrm{H}_{\mathrm{H}} 62_{\text {abc }}$ | 1,586 | 219 aAtCCGTGACATCATTGCTGCGCTCTGGTTCATTT |
|  |  | attattagacatattitatacattianactgtattacanattitcca CATTACTGGAGCTGCAGGGATCCCGAATTCTATTGGATCAGCGAGA <br>  tgGtgTanatctggactctanttccgtantatatcacgatacatcg taa anccgacactaanacgtccagacctacaatcactcgatcaa |
| PseB ${ }_{\text {HII }} 63_{\text {ab }}$ | 233 | 233 ATT |
| $\mathrm{PseB}_{1 \mathrm{k}} 78$ | 779 | 25 Cattiggctgactitatatatgitt |
|  |  | CCTAACAATTCTCATCTCGTCATTATTTGTAACCATAGAGCATGAA tTACCTCTTGAAGTCATCAGTGAGAATTTACGACTGGTCAACAAAA gCACGTGATTCCCTAACGCACCCCCACCCCCATATTTGGCCGCATA Catagcaanaacgangtacagtgcattgctatanttcattantac atcataantcgtgangcacagcgitatancgaccaagatctacaa |
| PseB $\mathrm{B}_{1 /} 79_{\text {abc }}$ | 252 | 231 ATC |
|  |  | ancattattclaagtctggagtactgtanaagcaccttitgccatg |
| PseB ${ }_{\text {kı }} 83$ | 12,413 | 65 Ctcgitatgitataggcat |
| PseB $\mathrm{K}_{\mathrm{k}} 84$ | 12,176 | 17 Gtttcatgiganctict |
|  |  | gTtGTtatcgitataantcttatgrgacangtccgatgitgatgcti tcttanaagtcgacatgittcatgigancttctangtgitatanctt |
| PseB $\mathrm{B}_{\text {k }} 85$ | 12,239 | 133 atticlaatctataccanctgiatantttangttiaca |
|  |  | GTTCATGTGTGTATACTTTTACTCGAATGACAAATGTACAATATGTT ATTATTCATGTGCGAAAAAGGTTGTAAATAATTATACATTTTTAATG tGGAAAATGGCATCTGTCATGTGCGACTGCCACTTAGTATTTTGCG |
| Pse $\mathrm{B}_{\mathrm{kL}} 86$ | 11,914 |  |
|  |  | afangtaggtatgacatticgatgtcaantgantgaggattitatc tagaagttagatcgtanaantcgcclagaccatagacagatacce CTCACTGGCTCTCAAAAGTCACGTGGGGTCCATAAAGTTAGTTTTA TGGTTTGGGGAGTTGACAATGTACTATATATTTCCCGTTCTAGAAT |
| PseB ${ }_{\text {K1 }} 87_{\text {abccef }}$ | 10,865 | 201 gtaagtgacggttaac |
| PseB kL $^{89}$ | 10,359 | 31 gGGAAAAGATtTTTTGTCATTTATGCTCCCT |
| PseB ${ }_{\text {kL }} 90{ }_{\text {abcd }}$ | 8,221 | ataatgiggtttccatattgataggagtantctgcgctcatatctca 84 TCAATAATTCATTGAAGCAGGGATCATTTCGAGGTCA |
| Pse $\mathrm{B}_{\mathrm{KL}} 91$ | 6,830 | 19 cagagccegcttcagacca |
| Pse $\mathrm{B}_{\text {Kı }} 94$ | 5,703 | 39 angttratgiggtglgiattcctgiatactattattatt |
| PseB ${ }_{\text {KL }} 95$ | 1,593 | 20 atttitttactgittcitca |
|  |  | aAgTaAtGacctggacaaiattcaatatgaccgagcangcgatat gCattactatagangtcgcaagtgggagagccctctagangggat GAAACGCAGGTCAGCGCGTCTAACAAATATTAAAATGTACTGGGA |
| PseB $\mathrm{kL}^{102}{ }_{\text {ab }}$ | 3,824 | 195 attantacganaict |
|  |  | tCTATATATACCCTGTAGATCCGAATTTGTGTGAACAGAGAAGCGG 77 TCACAAATTCGTATCTAGGGGAGTATGTAGT |
| PseB ${ }_{\text {KL }} 104_{\text {abcde }}$ | 1,219 | 77 TCACAAATTCGTATCTAGGGGAGTATGTAGT |
| PseB ${ }_{\text {KL }} 105_{\text {abc }}$ | 621 | 75 gatclaancangcccagatttatctetg |
| PseB ${ }_{\text {KL }} 106$ | 441 | 13 ttitcatgancac |
| PseBkL 108 | 61 | aAacgagtanaggantaganataaattitagtatattittgtgtcc <br> 60 AATTCAAAGAAATT |



| Oryzias latipes medaka Hox Ba |  |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: |
| PFC | Dist to ${ }^{\prime}$ gene | Length Sequence |  |  |  |
| $\mathrm{OlaBA}_{A C} 3$ | 4,855 | 21 anttanatatttttcagtict |  |  |  |
| OlaBA ${ }_{\text {cG }} 5$ | 25,610 | 37 Aaccagtanctganctatatatahgtcatantanatt |  |  |  |
| $\mathrm{OlaBA}_{\text {cG }} 6$ | 19,300 | 16 agantagtatttanca |  |  |  |
|  |  | aAAACATTTTCAGTTTGATGCAAAGAATTTTTGCAAATAAAATT tTTCTATACTTTTTTTGCCTCTAACCCAAAGTATGTAATAATTAA aAAAAAAATAAAAAATTTTGCTTCACTGGATTTAGCCATTTTTG tGaGCATCAGCTGTGCAGCACAGCCTCGTTGTCTTTGTTGGAGC |  |  |  |
| OlaBA ${ }_{\text {cG }} 7$ | 9,969 | 199 TTATTGTTGTGCTCCTTGCCTG |  |  |  |
| OlaBA ${ }_{\text {GG }} 12$ | 8,237 | 23 gitagitagacctctgcattcag |  |  |  |
|  |  | afttaacgegctetctgattagcagcgaacccagcaggicgaa |  |  |  |
| OlaBA ${ }_{\text {cG }}{ }^{13}$ | 1,565 |  |  |  |  |
| OlaBA ${ }_{\text {cG }} 14$ | 1,260 | 26 AAAGCGGGTtTtitttictutittic |  |  |  |
|  |  | agcanagceagangananganaganangagigacgianatctca TAATGTCCCTCTTCTTCTGCTTTAACAAACTCGCGGCTGCACGC |  |  |  |
| OlaBA ${ }_{\text {cG }} 15$ | 101 | 101 GGatantgiccgaca |  |  |  |
| OlabA ${ }_{\text {cG }}{ }^{16}$ | 4,219 | 23 Gtttcancattittgitttrgt |  |  |  |
| OlaBA $\mathrm{cG}^{18}$ | 10,345 | 27 tttgttgagtaaciacaggigatcagt |  |  |  |
|  | 3,582 | 15 tgatctcttcatatt <br> CTGTTTTCCCCTTTGTTGATGTTCTCGCTTATTATGCATTGGATAA |  |  |  |
|  | 19,382 | 47 A |  |  |  |
| OlaBA ${ }_{\text {cG }} 30$ | 9,226 | agaanganacagcatticctittctittcttattgangcactic 63 ctttattggitattiattg |  |  |  |
| OlaBA GH $^{40}$ | 5,500 | 29 tgitatagcttatanatatagagaganaa |  |  |  |
| OlaBA Gh $^{42}$ | 7,066 | 26 atganagaggattccanacatgcaga |  |  |  |
| OlaBA ${ }_{\text {gr }} 43$ | 8,371 | 15 тстtccgittctcac |  |  |  |
|  | 3,377 | gCaAtaAAAGAATATGACGGCAATAAAAGTTTATAGCGTATAA 83 ATTTCTGAAGGTTAAGAACTAAACGGCTGTAAAGCAAACA |  |  | CCGTTCATGCCGCAGGGAGTCCGGCGCGCGCCGCCCGGTCTGC TGCTGCTGCGGCTCCAGGAGGGGTGGTGCTGCTGCTGCTGCTGG |
| OlaBA gr $^{45}$ |  |  | $\mathrm{OlaBA}_{\text {LM }} 124$ | 2,850 | 107 TGGTCGCCAGCAGCCGTGCT |
|  |  | ancatgcagtcgaattatttigattittcaanttcaagatacttc | OlaBA ${ }_{\text {LM }}{ }^{125}$ | 2,564 | 24 Ccaccgatgctgaggaagitang |
|  |  | CAATTCTCCAAAATACCAGATTTTCTTTGITTTGCGTGCGTAAA |  |  |  |
|  |  |  |  |  | TTTAATGGCTTTTCTCTGTCTCCTCCACCTGCAAAGCTCCTCATG |
|  |  | ACTTAAACAATTGCCCTGCAGTAGACCAAATTCTCCAGGCCAT |  |  | GCAGGCGCCATTTGTAATTTTATATCCCGGGATAAATCGCAGAG |
|  |  | 318 ATGGTCCCATCCATGTTTATTGGATGAGAAACAAAA |  |  | TGGACACCAAGGCCGAGCTCGTСССССССтСССТGССТСССТTC |
| OlaBA ${ }_{\text {GH }} 48$ | 1,753 |  |  |  | CTCTCTGTGTTGTTGTTTGGGCGGCCGTGTCTCTGTCACCGGCCT gTGTGAGTCGATTATTCAGGGAGGCGTCTAGTAAAGTTGGTGAT |
|  |  | CGCTGTGGTTTGCTCTGGCTGCTCACCGTCGCTCTTAGCAGCCG | OlaBA ${ }_{\text {LM }}{ }^{126}$ | 2,068 | 442 TGTATTAAGCTGTAGTCCTACATGCAAGGGGCA |
|  |  | gcagccgegcgectgitgtggctgtgaggiganacaccacaic |  |  | CTATTCATGTCACGGAGCCTTTCAGGGGagGaigatggattea |
|  |  | GACGGTCACCGACACCGGGGGTAGACCTGCATTGCCTGCCCATC |  |  | СтСTGTCATTTCTTTATTCTGTCTCTCTAACCTTTAAGGTCAGATT |
|  |  | cटCCCACCCGCCTTGGTCTCTGTGTGTTATTAGGTCTGCCACAA |  |  | gCaggGgaccgitg anaiacaccgtcacgicacctccggtac |
| OlaBA ${ }_{\text {GH }} 53$ | 235 | 212 AAAAACCAACAACACACACCTGAAAGCCACTCTGACTC | OlaBA ${ }_{\text {LM }}{ }^{127}$ | 203 | 146 CAAATGGTCTtCC |
| OlabA ${ }_{\text {H, } 54}$ | 9,435 | 31 anaigacaianaicatgacanacgtcatcag | OlabA ${ }_{\text {LM }} 128$ | 12,880 | 22 тсСtcctgcctacctacagcet |
| OlabA ${ }_{\text {H } 57}$ | 24,265 | 37 Atttctaigacgigcgicagacgacttigtaiaictg |  |  |  |
| OlaBA ${ }_{\text {Hs }} 60_{\text {ab }}$ | 28,111 | 24 Cahcatganactgcctatttatgi | $\mathrm{OlaBA}_{\text {LM }} 135$ | 204 | 41 CCTATTCATGTCACGGAGCCTTTCAGGGGAGGAAGATGGAT |
|  |  | atganganatgiantanattccttgitgitttatganaattrac AACTTTGTGATACAAGTTTATGAGTGGCCGCGCGTGGGGATTGG | OlaBA ${ }_{\text {MN }} 136$ | 9,069 | 20 aAtcaagagctatctittct |
| OlaBA ${ }_{\text {Hu }} 65_{\text {bc }}$ | 6,481 | 146 tTtrTATGATTTCCCCCAATCTGCGATAAACTAAGTGATAGCAGCAGTCGGACTGTCG |  |  | AGCTCTCCTGCCTGGTAGATGAAATAGTTGAGATTTTAATGGGC |
|  |  |  |  |  | agttangaaggtagttctatteattatgccgagttitctccecct gCaGGCAACACATGTTGTTGTCAGCAGAGCTGAGCTGTGTGCTG |
| $\mathrm{OlaBA}_{H \mathrm{HJ}} 7 \mathrm{O}_{\text {ab }}$ | 256 | 66 Actatanagcacaicanatcata |  |  | GGCTCAGGCCCTCCATCAGCTGGAGAGCGACAGAGAAATGCA |
|  |  | $45{ }_{\text {CTGAAATGCATCCCGAGCCGGGAAATCAACAAATCATAAATCA }}$ |  |  | ACGCTGCTCCACAAAAGCACAGAGAAAAACCACTGGACGGCC 270 TCCCACTCGCTGTCTACTCTTTTTGCTTCCTGCAGTA |
| OlaBA ${ }_{\text {Jk }} 75$ | 1,118 |  | OlaBA мn $^{137}$ | 8,843 | 270 TCCCACTCGCTGTCTACTCTTTTTTGCTTCCTGCAGTA |
|  |  | 71 GgTCAATTAACAGGCCTGAGTGATGA |  |  |  |
| $\mathrm{OlaBA}_{\mathrm{Jk}} 76$ | 676 |  |  |  | Aacaggattitataggcttanctctcagtcatgcctacatccaa |
|  |  |  |  |  | Cattangcteacticctttcacangcagagticccagctgitga |
|  |  | TGACATTTACATGTCAAACAGATGAGGGGTTTTATCTCCGCGTC |  |  | CgTGttcaancanacctgtgagaggacatgcanggcgcatttt |
|  |  |  |  |  | agtgiccttacctttcgcacatcatcgacacagggtaantacc |
|  |  | tGGCTCTCCTCTGGTCACGTGTGGTCCATAAAGTTAGTTTTATGG tTTTGGGGAGTTGACATTGTACTATATATTTCACATTCTAGAAA | OlaBA ${ }_{\text {MN }} 138$ | 8,486 | 199 Aagagttgagaganctattiatta |
|  |  |  |  |  | tcttcactittantgiganaicagGgtatatatattigancaaa |
| OlaBA ${ }_{\text {kL }} 87_{\text {bccof }}$ | 10,515 | 193 gcangigacgittraic | OlaBA ${ }_{\text {MN }} 139_{\text {acde }}$ | 8,186 | 54 ATGCatgtc |
| OlabA ${ }_{\text {kı }} 96$ | 8,524 | 14 Cattitgitttica |  |  |  |
| OlaBA ${ }_{\text {kı }} 91$ | 6,950 | 19 cagagcccgettcagacca |  |  |  |
|  |  |  |  |  | TCTGGGCATCATTGTTGCACTTAGAGTTTACATTTTGATGGTTAA agGttananaatatantantganatcttattttgangacgaiag |
|  |  | ACAGGGGAACGCGGGGTGACACGCCTCGCCTGTTAAACAAAG |  |  | GCCTTCCAATCAAAGCGTCTTTCACCAATGCGTGTTCAAGGGAA |
| OlaBA ${ }_{\text {kL }} 102_{\text {b }}$ | 4,567 | 118 agtgicclanagtcgcagattanttganaict |  |  | CATTCATATATAAATA TTTATTTGTTATAGCCAGTCTAAAAGGA |
| OlaBA ${ }_{\text {kL }} 103$ | 1,050 | 19 Aacattigggcatttattg | OlaBA Mr $^{140}{ }_{\text {abc }}$ | 7,951 | 260 gagGaianaadatactgTactittteagtatt |
|  | 1,310 | 71 AACAATCACACAAATTCGCTCTICTAGGGGGA | OlaBA ${ }_{\text {MN }} 141$ | 7,391 | 17 ancaattctttantaaa |
| OlaBA kL $^{104} 4_{\text {abcd }}$ |  |  |  |  | GCTGCTGTCAGCTCTCTCCAAGAGAAAGATTGATCACCGCATTT |
|  |  | gTGAttTagGaggccgigcgiccagaatcacttgatganttic | OlaBA ${ }_{\text {N1 }} 142$ | 5,129 | ( СтTтTCTCCCACTCGGGGCTCCTGTGGCCCTCCTCAAAACTTCC |
| OlaBA ${ }_{\text {kL }} 105_{\text {ac }}$ | 682 | 76 TATCGATCCTAAACAAGCCAGATTCATCTCTG | $\mathrm{OlaBA}_{\text {MN }} 142$ |  |  |
|  |  |  |  |  | Catattgtanacgatactggccagctectetcgcctgatgitac |
|  |  |  |  |  | CGGCCATTACATCAGCCCAGAGGAGTGGAGCAAATGCCCATTG |
|  |  | gccttgagtgatcgcgatacattacgcgcagtantcttgttta |  |  | TTTGGGCCTGTCATCGGCTGGGATGGGCGGACTTGCCGCCCACA |
|  |  | AATCACAAGCCTTCCCCCCGTTTCACTCCCGTTCTCCCGTTAAT |  |  | CGGGGCACATGGCGACCGGTCGCCTTCTCCCTTTCTTGCCCCTC |
| OlaBA ${ }_{\text {LM }} 109$ | 26,025 | 190 ttancggcagctcag | OlaBA ${ }_{\text {MN }} 143$ | 4,504 |  |
|  |  | ATAGCTCAAACGTTGACAACCAAATAGATAATCAACAAGACG |  |  | tttagacctragttattrattgagattcttrantagtganaitc |
| OlaBA ${ }_{\text {LM }} 113_{\text {bc }}$ | 256,339 |  |  |  | CaAATTATTTATTGTACATATATTTTCATAGTGGAATAATAATA |
| Olabi ${ }_{\text {LM }} 118_{\text {b }}$ | 256,339 18,643 | 26 CTGACGTGGAAAATtCAACTGTCCTT | OlaBA ${ }_{\text {No }} 147$ | 8,175 |  |
| OlaBA ${ }_{\text {LM }} 119$ | 11,949 | 29 ttatttanatatanacanaacgtanatat | $\mathrm{OlaBA}_{\mathrm{NO}} 154_{\mathrm{ab}}$ | 8,161 | 62 tacatatattitcatag |
| OlabA ${ }_{\text {LM }} 120$ | 7,000 | 24 tgtaitgitcattaaatttanatg |  |  | CTGCTCCCCTCCAGAGTCAAAATGGCGCTGGAGACAAAGGAGG |
|  |  |  | OlaBA ${ }_{\text {No }} 148$ | 7,478 | 72 atgigaicat |
|  |  | GATGTGTAAGATGAGAACCAGAATCAATAAGAGCTCATATCGC | OlaBA ${ }_{\text {NO }} 149$ | 7,096 | 17 atttatctctgcattaa |
|  |  | TCCCACTCAGCCTCCATCCTCTCTGCTGCTTTTTGCTTTTATTCC GGGAATAATTCCAGGTCAGCAGTACCTGTCAGCGGCTGCTCCT | OlaBA $_{\text {NO }} 155$ | 961 | 30 aggttctctattanactggcagaittatg |
|  |  | GTCCCACAACCTTCGATTAGACTACAAGAGGTTGGTGCTCCCTA |  |  |  |
|  |  | TCTGATTTTTCAGCCCCTGCATCTTTCTTCCCTTTCTTTTCCGCAT |  |  |  |
| $\mathrm{OlaBA}_{\text {LM }} 121$ | 5,846 | 227 tttatc |  |  | GCTGATATCAGATTGATGGGCCGGTTTGATTGAAGTCTCTTTGTC |
| $\mathrm{OlaBA}_{\text {LM }} 122$ | 5,232 | 14 actcgtgtaitgra |  |  | gCGCTGATGTCACTGCGAGTGATGGATGAGGCGCGTGCGCCAC TATTGACGCGCGCGCGCACCAACTTCACTCCTGAGCCGAAAAC |
|  |  |  | $\begin{aligned} & \text { OlaBA }_{\text {NO }} 156 \\ & \text { OlaBA }_{\mathrm{NO} 161_{\text {abc }}} \\ & \text { OlaBA }_{\text {NO }} 160 \end{aligned}$ |  | TATTGACGCGCGCGCGCACCAACTICACTCCTGAGCCGAAAAC GACCCCATCCCCTCCTCCTGTACGCACACACACACACAACAC |
|  |  | gGagaaccgcgagaacgatcagGagangaacggctgatcaca |  | 740 | 288 TGCCGTGACCAGCTGACAACTTCTGGCCCGGTCACCA |
|  |  | GAAAATGGGATGGGGTAACGGTTCCGTCTGGATCTTTGTCTGAG |  |  |  |
|  |  | ATTCTCGGCCCGGATGGCTGCCGGCAGAGCCGCTCAAGCCAGA gaaattcgaggccattgitatcagccagcagcggctgcagcca |  | 687 | 64 atcicactgcgactgatgia |
| OlaBA ${ }_{\text {LM }} 123$ | 3,199 | 207 GAGCTGGTGTCGGGGCGGAGGGTGGAGGTGGAGGG |  | 812 | 21 сстСстCacgiacgitccttt |




| PFC | Coelacanth C | Frog C | Human C | Zebrafish Ca | Medaka C | Tetraodon C | Zebrafish Cb |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| 1 |  |  |  |  | $\mathrm{OlaC}_{\mathrm{CD}} 1$ | $\mathrm{TniC}_{\text {CD }} 1$ |  |
| 2 |  |  |  | DreCA ${ }_{\text {cD }}{ }^{2}$ | $\mathrm{OlaC}_{\mathrm{CD}}{ }^{2}$ | $\mathrm{TniC}_{\mathrm{CD}} 2$ |  |
| 3 |  |  |  |  | $\mathrm{OlaC}_{\mathrm{CD}} 3$ | $\mathrm{TniC}_{\text {cD }} 3$ |  |
| 4 |  |  |  | DreCA ${ }_{\text {CD }} 4$ | $\mathrm{OlaC}_{\text {cD }} 4$ |  |  |
| 5 |  |  |  | DreCA ${ }_{\text {cD }} 5$ | $\mathrm{OlaC}_{\mathrm{CD}} 5$ | $\mathrm{TniC}_{\text {cD }} 5$ |  |
| 6 |  |  |  |  | $\mathrm{OlaC}_{\text {cD }} 6$ | $\mathrm{TniC}_{\text {cD }} 6$ |  |
| 7 |  |  |  | DreCA ${ }_{\text {CD }} 7$ |  | $\mathrm{TniC}_{\text {cD }} 7$ |  |
| 8 | $\mathrm{LmeC}_{\mathrm{CD}} \mathrm{c}_{\text {cle }}$ | $\mathrm{XtrC}_{\mathrm{CD}} 8_{\mathrm{d}}$ | $\mathrm{HsaC}_{\text {cD }} \mathrm{D}_{\text {bde }}$ | DreCA ${ }_{\text {cD }} 8_{\text {abcde }}$ | $\mathrm{OlaC}_{\mathrm{CD}} 8_{\text {abcede }}$ | $\mathrm{TniC}_{\mathrm{CD}} 8_{\text {abcde }}$ |  |
| 9 | $\mathrm{LmeC}_{\mathrm{CD}} 9$ |  | $\mathrm{HsaC}_{\mathrm{CD}} 9$ |  |  |  |  |
| 10 | $\mathrm{LmeC}_{\mathrm{CD}} 10$ |  | $\mathrm{HsaC}_{\mathrm{CD}} 10$ |  |  |  |  |
| 11 | $\mathrm{LmeC}_{\text {DE }} 11$ |  | $\mathrm{HsaC}_{\mathrm{DE}} 11$ |  |  |  |  |
| 12 | $\mathrm{LmeC}_{\text {DE }} 12$ |  | $\mathrm{HsaC}_{\text {DE }} 12$ |  |  |  |  |
| 13 |  |  |  |  | $\mathrm{OlaC}_{\text {DE }} 13$ | $\mathrm{TniC}_{\text {DE }} 13$ |  |
| 14 |  |  |  |  | $\mathrm{OlaC}_{\text {DE }} 14$ | $\mathrm{TniC}_{\text {DE }} 14$ |  |
| 15 |  |  |  |  | $\mathrm{OlaC}_{\text {DE }} 15$ | Tnic ${ }_{\text {DE }} 15$ |  |
| 16 |  |  |  |  | $\mathrm{OlaC}_{\text {DE }} 16$ | TniC CE 16 |  |
| 17 |  |  |  |  | $\mathrm{OlaC}_{\text {DE }} 17$ | $\mathrm{TniC}_{\text {DE }} 17$ |  |
| 18 |  |  |  |  | $\mathrm{OlaC}_{\text {DE }} 18$ | $\mathrm{TniC}_{\text {DE }} 18$ |  |
| 19 |  |  |  |  | $\mathrm{OlaC}_{\text {DE }} 19$ | $\mathrm{TniC}_{\text {DE }} 19$ |  |
| 20 |  |  |  |  | $\mathrm{OlaC}_{\text {DE }} 20$ | $\mathrm{TniC}_{\text {DE }} 20$ |  |
| 21 |  |  |  |  | $\mathrm{OlaC}_{\text {DE }} 21$ | $\mathrm{TniC}_{\text {DE }} 21$ |  |
| 22 |  |  |  | DreCA ${ }_{\text {dE }} 22$ | $\mathrm{OlaC}_{\text {DE }} 22$ |  |  |
| 23 |  |  | $\mathrm{HsaC}_{\text {DE }} 23$ |  |  |  | $\mathrm{DreCB}_{\text {DE }} 23$ |
| 24 | $\mathrm{LmeC}_{\text {DE }}{ }^{24}$ |  | $\mathrm{HsaC}_{\text {DE }} 24$ |  |  |  |  |
| 25 |  | XtrC ${ }_{\text {DE }} 25$ | $\mathrm{HsaC}_{\text {DE }} 25$ |  |  |  |  |
| 26 | $\mathrm{LmeC}_{\mathrm{E}} 26_{\text {abc }}$ | $\mathrm{XtrC}_{\text {DE }} 22_{\text {abode }}$ | $\mathrm{HsaC}_{\text {DE }} 26_{\text {abcde }}$ | DreCA ${ }_{\text {DE }} 26_{\text {bode }}$ | $\mathrm{OlaC}_{\text {DE }} 26_{\text {bcd }}$ | TniC $^{\text {DE }} 26_{\text {bcd }}$ | DreCB ${ }_{\text {DE }} 26^{\text {b }}$ |
| 27 |  |  |  |  | $\mathrm{OlaC}_{\text {EF }} 27$ | $\mathrm{TniC}_{\text {EF }} 27$ |  |
| 28 |  |  |  | $\mathrm{DreCA}_{\text {EF }} 28$ | $\mathrm{OlaC}_{\text {EF } 28}$ | $\mathrm{TniC}_{\text {EF2 }} 28$ |  |
| 29 |  |  |  | DreCA $^{\text {EF } 29}$ | $\mathrm{OlaC}_{\text {EF2 }} 29$ | $\mathrm{TniC}_{\text {EF }}{ }^{29}$ |  |
| 30 |  |  |  | $\mathrm{DreCA}_{\text {er }} 30$ | $\mathrm{OlaC}_{\text {EF }} 30$ | TniC ${ }_{\text {EF }} 30$ |  |
| 31 |  |  |  | $\mathrm{DreCA}_{\text {EF }} 31$ |  | $\mathrm{TniC}_{\text {EF }} 31$ |  |
| 32 |  |  | $\mathrm{HsaC}_{\text {EFF }} 32$ |  |  |  | DreCB ${ }_{\text {EJ }} 32$ |
| 33 |  | XtrC ${ }_{\text {EF }} 33$ | $\mathrm{HsaC}_{\text {EF }} 33$ |  |  |  |  |
| 34 |  | XtrC EFF 3 | $\mathrm{HsaC}_{\text {EFF }} 34$ |  |  |  |  |
| 35 | $\mathrm{LmeC}_{\text {EF }} 35_{\text {abc }}$ | $\mathrm{XtrC}_{\text {EF }} 35_{\text {ab }}$ | $\mathrm{HsaC}_{\text {EF }} 35_{\text {ab }}$ | $\mathrm{DreCA}_{\text {EF }} 35_{\text {abc }}$ | $\mathrm{OlaC}_{\text {EF }} 35_{\text {ac }}$ | TniC ${ }_{\text {EF }} 35_{\text {ac }}$ |  |
| 36 | $\mathrm{LmeC}_{\mathrm{FG}} 36$ | $\mathrm{XtrC}_{\mathrm{FG}} 36$ | $\mathrm{HsaC}_{\mathrm{FG}} 36$ |  |  |  |  |
| 37 |  |  |  |  | $\mathrm{OlaC}_{\mathrm{FG}} 37$ | $\mathrm{TniC}_{\text {FG }} 37$ |  |
| 38 | $\mathrm{LmeC}_{\mathrm{FG}} 38_{\mathrm{ab}}$ | $\mathrm{XtrC}_{\mathrm{FC}} 38_{\mathrm{a}}$ | $\mathrm{HsaC}_{\mathrm{Fc}} 38_{\mathrm{ab}}$ | DreCA $_{\text {FG }} 38_{\text {ab }}$ | $\mathrm{OlaC}_{\mathrm{FG}} 38_{\mathrm{ab}}$ | $\mathrm{TniC}_{\mathrm{FG}} 38_{\mathrm{ab}}$ |  |
| 39 |  |  |  |  | $\mathrm{OlaC}_{\mathrm{FG}} 39$ | $\mathrm{TniC}_{\text {FG }} 39$ |  |
| 40 | $\mathrm{LmeC}_{\text {FG }} 40_{\text {abc }}$ | $\mathrm{XtrC}_{\mathrm{FG}} 40_{\mathrm{abc}}$ | $\mathrm{HsaC}_{\text {FG }} 40_{\mathrm{ac}}$ | DreCA ${ }_{\text {FG }} 40{ }_{\text {bcd }}$ | $\mathrm{OlaC}_{\text {FG }} 4 \mathrm{ob}_{\text {bed }}$ | $\mathrm{TniC}_{\text {FG }} 4 \mathrm{l}_{\text {bcd }}$ | DreCB ${ }_{\text {FG }} 40 \mathrm{abc}_{\text {abe }}$ |
| 41 |  |  |  |  | $\mathrm{OlaC}_{\mathrm{FG}} 41$ | $\mathrm{TniC}_{\text {cG }} 41$ |  |
| 42 |  |  |  |  | $\mathrm{OlaC}_{\text {FG }} 42$ | $\mathrm{TniC}_{\mathrm{FG}} 42$ |  |
| 43 |  |  | $\mathrm{HsaC}_{\mathrm{FG}} 43$ | DreCA ${ }_{\text {FG }} 43$ |  |  |  |
| 44 |  |  |  | $\mathrm{DreCA}_{\text {FG }} 44$ |  | $\mathrm{TniC}_{\text {FG }} 44$ |  |
| 45 |  |  |  | $\mathrm{DreCA}_{\text {FG }} 45$ |  | $\mathrm{TniC}_{\mathrm{FG}} 45$ |  |
| 47 |  |  | $\mathrm{HsaC}_{\mathrm{FG}} 47$ |  |  |  | DreCB ${ }_{\text {EJ }} 47$ |
| 48 |  | $\mathrm{XtrC}_{\mathrm{FG}} 48$ | $\mathrm{HsaC}_{\mathrm{FG}} 48$ |  |  |  |  |
| 49 |  | XtrC ${ }_{\text {FG }} 49$ | $\mathrm{HsaC}_{\mathrm{FG}} 49$ |  |  |  |  |
| 50 | $\mathrm{LmeC}_{\mathrm{FG}} 50_{\mathrm{ab}}$ | $\mathrm{XtrC}_{\mathrm{FG}} 50_{\mathrm{a}}$ | $\mathrm{HsaC}_{\mathrm{FG}} 50_{\mathrm{ab}}$ | DreCA $_{\text {FG }} 50_{\mathrm{ab}}$ |  | $\mathrm{TniC}_{\mathrm{FG}} 50_{\mathrm{ab}}$ |  |
| 51 |  | $\mathrm{XtrC}_{\mathrm{FG}} 51$ | $\mathrm{HsaC}_{\mathrm{FG}} 51$ |  |  |  |  |
| 52 | $\mathrm{LmeC}_{\mathrm{FG}} 52_{\text {abc }}$ | $\mathrm{XtrC}_{\mathrm{FG}} 52_{\text {bc }}$ | $\mathrm{HsaC}_{\text {FG }} 52_{\text {abc }}$ | DreCA ${ }_{\text {FG }} 52_{\text {c }}$ | $\mathrm{OlaC}_{\text {FG }} 52_{\text {abc }}$ | $\mathrm{TniC}_{\text {FG }} 52_{\text {abc }}$ |  |
| 53 |  |  |  |  | $\mathrm{OlaC}_{\mathrm{GH}} 53$ | $\mathrm{TniC}_{\text {GH }} 53$ |  |
| 54 |  |  | $\mathrm{HsaC}_{\mathrm{GH}} 54$ |  |  |  | $\mathrm{DreCB}_{\text {EJ }} 54$ |
| 55 | $\mathrm{LmeC}_{\text {GH }} 55$ | $\mathrm{XtrC}_{\mathrm{GH}} 55$ | $\mathrm{HsaC}_{\text {GH }} 55$ |  |  |  |  |
| 56 |  |  |  | DreCA ${ }_{\text {GH }} 56$ | $\mathrm{OlaC}_{\text {GH }} 56$ | $\mathrm{TniC}_{\text {GH }} 56$ |  |
| 57 | $\mathrm{LmeC}_{\text {GH }} 57$ |  | $\mathrm{HsaC}_{\text {GH }} 57$ |  |  |  |  |
| 58 | $\mathrm{LmeC}_{\text {GH }} 58$ |  | $\mathrm{HsaC}_{\mathrm{H} 5} 58$ |  |  |  |  |
| 59 |  |  |  |  | $\mathrm{OlaC}_{\text {GH }} 59$ | $\mathrm{TniC}_{\text {GH }} 59$ |  |


| 60 |  |  |  |  | $\mathrm{OlaC}_{\text {GH }} 60$ | $\mathrm{TniC}_{\text {GH }} 60$ |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| 61 |  |  |  |  | $\mathrm{OlaC}_{\text {GH }} 61$ | $\mathrm{TniC}_{\text {GH }} 61$ |  |
| 62 |  |  |  |  | $\mathrm{OlaC}_{\text {GH }} 62$ | $\mathrm{TniC}_{\text {GH }} 62$ |  |
| 63 |  |  |  |  | $\mathrm{OlaC}_{\text {GH }} 63$ | $\mathrm{TniC}_{\text {GH }} 63$ |  |
| 64 |  |  |  |  | $\mathrm{OlaC}_{\mathrm{GH}} 64$ | $\mathrm{TniC}_{\text {GH }} 64$ |  |
| 65 |  |  |  | $\mathrm{DreCA}_{\text {HJ }} 65$ | $\mathrm{OlaC}_{\text {HJ }} 65$ |  |  |
| 66 |  |  |  |  | $\mathrm{OlaC}_{\mathrm{HJ}} 66$ | $\mathrm{TniC}_{\text {H }} 66$ |  |
| 67 | $\mathrm{LmeC}_{\text {HJ }} 67{ }_{\text {b }}$ | $\mathrm{XtrC}_{\text {HJ }} 67 \mathrm{~b}$ | $\mathrm{HsaC}_{\mathrm{HJ}} 67{ }_{\mathrm{b}}$ | DreCA ${ }_{\text {HJ }} 67{ }_{\text {abc }}$ | $\mathrm{OlaC}_{\mathrm{HJ}} 67{ }_{\text {abc }}$ | $\mathrm{TniC}_{\mathrm{HJ}} 67_{\text {abc }}$ | $\mathrm{DreCB}_{\mathrm{EJ}} 67 \mathrm{~b}$ |
| 68 |  |  |  |  | $\mathrm{OlaC}_{\mathrm{HJ} 68}$ | $\mathrm{TniC}_{\mathrm{HJ} 68}$ |  |
| 69 |  |  |  |  | $\mathrm{OlaC}_{\mathrm{HJ}} 69$ | $\mathrm{TniC}_{\mathrm{HJ} 69}$ |  |
| 70 |  |  |  |  | $\mathrm{OlaC}_{\text {H, }} 70$ | $\mathrm{TniC}_{\text {H }} 70$ |  |
| 71 |  |  |  |  | $\mathrm{OlaC}_{\mathrm{HJ}} 71$ | $\mathrm{TniC}_{\text {HJ }} 71$ |  |
| 72 |  |  |  |  | $\mathrm{OlaC}_{\mathrm{HJ}} 72$ | $\mathrm{TniC}_{\text {HJ } 72}$ |  |
| 73 |  |  |  |  | $\mathrm{OlaC}_{\mathrm{HJ}} 73$ | $\mathrm{TniC}_{\text {H } 73}$ |  |
| 74 |  |  | $\mathrm{HsaC}_{\mathrm{H} 7} 74$ | $\mathrm{DreCA}_{\mathrm{HJ}} 74$ |  |  |  |
| 75 | $\mathrm{LmeC}_{\mathrm{H} 7} 75$ |  | $\mathrm{HsaC}_{\mathrm{H} 7} 75$ |  |  |  |  |
| 76 |  |  | $\mathrm{HsaC}_{\mathrm{HJ} 76}$ |  |  |  | DreCB ${ }_{\text {EJ }} 76$ |
| 77 | $\mathrm{LmeC}_{\mathrm{H} 7} 77$ |  | $\mathrm{HsaC}_{\mathrm{H} 77} 7$ |  |  |  |  |
| 78 | $\mathrm{LmeC}_{\mathrm{H} 7} 78$ | $\mathrm{XtrC} \mathrm{HJ}^{7} 8$ | $\mathrm{HsaC}_{\mathrm{HJ} 7} 7$ |  |  |  |  |
| 79 | $\mathrm{LmeC}_{\mathrm{H} 7} 79$ |  | $\mathrm{HsaC}_{\mathrm{H} 7} 79$ |  |  |  |  |
| 80 | $\mathrm{LmeC}_{\mathrm{HJ}} 80$ |  | $\mathrm{HsaC}_{\mathrm{HJ}} 80$ |  |  |  |  |
| 81 | $\mathrm{LmeC}_{\mathrm{H} 8} 81$ |  | $\mathrm{HsaC}_{\mathrm{HJ} 81}$ |  |  |  |  |
| 82 |  |  |  |  | $\mathrm{OlaC}_{\text {JK }} 82$ | TniC ${ }_{\text {IK }} 82$ |  |
| 83 |  |  |  | $\mathrm{DreCA}_{\text {JK }} 83$ | $\mathrm{OlaC}_{\text {JK }} 83$ | $\mathrm{TniC}_{\text {JK }} 83$ |  |
| 84 |  |  |  |  | $\mathrm{OlaC}_{\mathrm{KL}} 84$ | $\mathrm{TniC}_{\mathrm{KL}} 84$ |  |
| 85 |  |  |  |  | $\mathrm{OlaC}_{\text {Kı }} 85$ | TniC ${ }_{\text {Kı }} 85$ |  |
| 86 |  |  |  |  | $\mathrm{OlaC}_{\text {kL }} 86$ | $\mathrm{TniC}_{\text {Kı }} 86$ |  |
| 87 | $\mathrm{LmeC}_{\text {K1 }} 87_{\text {abcd }}$ | XtrC ${ }_{\text {KL }} 87_{\text {abcd }}$ | $\mathrm{HsaC}_{\mathrm{KL}} 87_{\text {abcd }}$ | DreCA ${ }_{\text {KL }} 87_{\text {bc }}$ | $\mathrm{OlaC}_{\text {KL }} 87_{\text {ac }}$ | $\mathrm{TniC}_{\text {KL }} 87_{\text {ac }}$ |  |
| 88 | $\mathrm{LmeC}_{\mathrm{KL}} 88$ |  | $\mathrm{HsaC}_{\text {KL }} 88$ |  |  |  |  |
| 89 | $\mathrm{LmeC}_{\mathrm{KL}} 89$ |  | $\mathrm{HsaC}_{\mathrm{KL}} 89$ |  |  |  |  |
| 90 | $\mathrm{LmeC}_{\mathrm{KL}} 90$ |  | $\mathrm{HsaC}_{\mathrm{KL}} 90$ |  |  |  |  |
| 91 | $\mathrm{LmeC}_{\mathrm{KL}} 91$ |  | $\mathrm{HsaC}_{\mathrm{KL}} 91$ |  |  |  |  |
| 92 |  |  |  |  | $\mathrm{OlaC}_{\mathrm{KL}} 92$ | TniC ${ }_{\text {Kı }} 92$ |  |
| 93 |  |  |  | DreCA $_{\text {KL }} 93$ |  | $\mathrm{TniC}_{\text {Kı }} 93$ |  |
| 94 |  |  |  |  | $\mathrm{OlaC}_{\text {Kı }} 94$ | TniC ${ }_{\text {Kı }} 94$ |  |
| 95 |  |  |  |  | $\mathrm{OlaC}_{\text {Kı }} 95$ | TniC ${ }_{\text {Kı }} 95$ |  |
| 96 |  |  |  | $\mathrm{DreCA}_{\text {KL }} 96$ | $\mathrm{OlaC}_{\text {Kı }} 96$ |  |  |
| 97 |  |  |  |  | $\mathrm{OlaC}_{\text {Kı }} 97$ | $\mathrm{TniC}_{\mathrm{KL}} 97$ |  |
| 98 |  |  |  | $\mathrm{DreCA}_{\text {KL }} 98$ |  | TniC KLI 98 |  |
| 99 |  | $\mathrm{XtrC} \mathrm{KL}^{99} \mathrm{a}_{\text {ab }}$ | $\mathrm{HsaC}_{\mathrm{KL}} 99_{\mathrm{ab}}$ | DreCA ${ }_{\text {KL }} 99{ }_{\mathrm{b}}$ | $\mathrm{OlaC}_{\mathrm{KL}} 99^{\text {ab }}$ | $\mathrm{TniC}_{\mathrm{KL}} 99^{\text {ab }}$ |  |
| 100 |  | $\mathrm{XtrC} \mathrm{C}_{\text {LL }} 100$ | $\mathrm{HsaC}_{\mathrm{KL}} 100$ |  |  |  |  |
| 101 |  | $\mathrm{XtrC}_{\mathrm{KL}} 101$ | $\mathrm{HsaC}_{\text {KL }} 101$ |  |  |  |  |
| 102 | $\mathrm{LmeC}_{\text {KL }} 102$ | $\mathrm{XtrC} \mathrm{KLL}^{102}$ | $\mathrm{HsaC}_{\mathrm{KL}} 102$ | DreCA ${ }_{\text {KL }} 102$ | $\mathrm{OlaC}_{\mathrm{KL}} 102$ | $\mathrm{TniC}_{\text {KL }} 102$ |  |
| 103 |  |  |  | DreCA ${ }_{\text {LM }} 103$ | $\mathrm{OlaC}_{\text {LM }} 103$ |  |  |
| 104 |  |  |  | DreCA ${ }_{\text {LM }} 104$ | $\mathrm{OlaC}_{\text {LM }} 104$ |  |  |
| 105 |  |  |  | DreCA ${ }_{\text {LM }} 105$ | $\mathrm{OlaC}_{\text {LM }} 105$ |  |  |




 410

位位TTCCTCTAAAACAAATGCTTCTAAACTTG AAAATCCACGTTGTGTTTATCGGCATTTAGCACTAATGTTCAAGCTG TGAGCTAAAAGCTACAGTTTCGCCTCAACTCCTGCGCCTCAATTG GCTGAGAAGGGTCAGCTGACACTGTCATA
TAACTACCTAAAAAAACTACCTAAAAATGTATGGACTTGAGTGCG
ACTTCACTGCATGATGGCTTCCCCGCTGCTCTTATCTCTTGTG ACTTCACTGCATGATGGCTTCCCCGCTGCTCTTTATCTCTTGTTGAC

TGACGCTCTTGTCTAACTGTCCTGCCCTATTTGCGCCAAAGAACCC TGCCTGGCCCCTAACAACTAACAGCTCAAAGTTTACCGGACACGI ACTGGCCCAACACGTGCACGTGATCACATACAAATACTCCCATATATTI gGGCaGCGCAAGCCGGATCAAGAATGCAAAAAAAAAAAAAAAAA
CCTTGTTTTCTCAGCTCCCTCATTATGGCTTTAGTACCGCTGGAAG ACAGTCTAGAATCCAAAGTCACTTTCATTCAGGCCCCTGAATGAG AAACAGGCTGTAAGAGCAGCACAGTTTCACCCTAAAACCATAGG CACATAAAGTCATTTATTTCACTTTGTTGTGGCGCACAGGAACTGA 398 AGCCGCAGGACAGACACTTTCTCGACGCA
tattancgacacgittitgantantcanatgatcctcgitananti tattgitggatatanaiancatgantggagagctgicgaitatag 112 TGCGCTGTAGTGGAGCTGCTT
gCGCATTGATCCACTGTACAATTTTTGIGGGGGIAAATATAATCAC GTGTCAGCGAGGCAGCCAATAGGAGCCCGGGAAGCTCTGAGAAA TAATTACCTGCCTIGATTGTCCTATGGCCAGATAAAAAAGTACAC

13 ttgtticattigt
aAattgcctuttrgiggtggcaaicaatacaagcaggatgccata ATTTCAAAGCCCAATCCCGTGCATTTCCGCTCATCCCGGACAAAA ATGCCAGTTTTACGGCCCCTGTTGGAGCTCGGCTGTTGGTCTCAAA TGCAGACGAACAAAGCAAACTCGACTAACTGGCTAGACGTCTGG

CTAGATGACGCTGTTGGTCCGTGTTTTGAGCGAAGCCTGACTGTTG GCGGACTTTTTTAACTGCTTTGTGGCTTGGCTGAAGAATGAAAAGT 5 tgtgTantcgaccacaga
35 aCattitacaccttaaccccgattctcattgaat
CTATGTTTTATAAAAATGCGTAAAAAAACGCAGGGAACCCCAGA AAAACCACGCAAGCCACCGCTTTTGCATGTCAGGAGCAAAGTTGI CATTTTATGAACTCCCGTGTGGGAGATTTACTGCGTCTCCTCGCCG 224 GTTTTACGGGGTCAGTTAGTGGCACACGGTATTCATAGGTCGTG
tTATGATTAGTGAAGCTTTGCTCAAGGTGGTAATACGCAGGGCCC GCGTCTGACCGGTCATGGAAAGATGAAGGGGTGGGGAAGGTGGA GAAAGAAAAGAGCAGCGTCCGTGACTGTGCCGGTGGGACAATCC actctcacgccatgac 451 CTGCTTTTGAGACTGCAAAGCCCGAAGAAGAAC

TTTTATTGTCCTTTTTATGGTTATATGGCTGAAAAGAGAAAAAGAA GGCTCCCATAAACAGAGCAAGAGTCACAAGGAGGCAGTGAGCG 170 CaAaAAAATAAATAAATAAATCCTGCTTCATAAA TCAGACATCAGAATTTTTTTCTAGGATTACCAGGGAAACGCAACA GTTTGAAATCCTTTGATGCTACCTAAACCTGGGGAAATTGCAATA Gagattictgantatagagatticatatggtttaanacattittta
taAacactigitcaagacaattitttggagtctigggacgacaag TAGCTGAAAGCCAAGCGAACAATTGAGAGATTTTACCACGGCACT ACCTAATCTCAGAGCTTTGCCTTTGGGGAGTTTACCTTCCGCACAA GTGCGGCATCGCTCCGACGTGATTGCAAGCTATATTTCCCCAACA tacaacgictitaangtgaggtcaattra

GTGTGTATATACTTTTACTAGTGAGTTAACATTACCAATAAAGTTT AGTGTACCTGCAGTAAACTTTATTGGGGTGTAAAGACATGGGTCA 174 aAAACGGCCTGCGAGGATTCCCCGCATTTCTAGTGT

ACATtCCTGGitGTtaAaGGAGAGAAATtTACAGCTGAGTAATAA AAGTTTACGACTGAATCCTTCCTACGATTGGCTGCGGCGAGCCAC
GTGGCGCACGCTCTGTGAACATGAACTTTATGCTGTTGTCT

TGACCTAACCTGTAGCCTGTATATAGATATGCTGATGCTGGGAGC GGTTGTCATGGTTCCTCATTGTTTGGAGCACCTGCGTGCGGCTGGC ATCGTGGGGAAAACATTTCACAAGAGCTGTGCTAATCTGTTGTAG TCATCACTCATAGGCCCTT

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CTCCATGAAAGTCGTGAAGCTGTCTGCGTCACCACATTTAGAAAC CTCCATGAAAGTCGTGAAGCTGTCTGCGTCACCACATTTAGAAAC gTCATAATTTATGACGTGCGGATCAACATGTGCTCATTGGGCAGC 349 AGCTTCCTGAGTGCTTAACGAGCATGAAGT

ATAAAACACTAAAACTCTGGGGAAAGAATGACCTTTCTGATGCTG CACACGGACACAGCCTCTGCTGCATTCCAGTCCACACATTGGCTI TTTTCGTGCGTGTGTGTGTTTGAGAGAGAAACGAGCCTGAAAACA AAACACACATATTTGCTTTCAGTTTTCACACTTTTGATTGTATTATI 6 AAACACAC
GCTTTCATCTGTGCGTCCGGATGATAGCCTTGAGCTGCTCCGCTGC AGAGATAGCTCTTGGTGAGCTGTGGAACGTTACTTGTTTTATTGCC GCCCCCAAAAAGCTGTGCTCACTTGACAGATAAATCATACACAA 193 agGacacattaa

55 Gataacaangctcactccacctataatcc

|  |  | gCaggagtgccgcctgaccttcagatganccgcaaggagcaitg aCATCATGACACCGCTCTGTGTGCTTCATCACCAATTATGTGTCAC TTGCTGCAGTTTGGGACCTAATGTATTGAATATCAATCACTTTGAT |
| :---: | :---: | :---: |
| OlaC ${ }_{\text {kL }} 84$ | 8,578 | 217 ACCCCACTTTACTGCGTGTAAGGAACAGGAGGTTG |
|  |  | aAtgTgTgTGGACTGACCAGTGTGCCTGTAAGTGGATTCTCCGTCT GTGTCTGTCATTCCTCGTGGAAGTGAATCATTCTGTAAACTGTATT CTGACGGTGCCTGCTAAACACAAGGCCTCAAAAATCACTGAAAG |
| OlaC ${ }_{\text {KL }} 85$ | 8,134 | 215 atagcctatttgitgactgicattantanagtt |
|  |  | GACAAACGCGCTGAGAAGTTTAAAGCCCCCCCATAAAACTTTATT GCCCCTTITTCCATTACTCCGCAGGCACACTGCGCTTCCTGTTTTG CCAGGGAAGGAAGGGACCCACAATCCTGTAATAACAACACACAT |
| OlaC $\mathrm{kLI}^{8} 8$ | 7,734 | 146 Ggacgcaaial |
|  |  |  ATGGGGAGCACGTGGTGTCATTTAAGTGGGTTTTATGGCCTGGAA gagctancanaccttcgatatatacacatcatatataitcttaic |
| OlaC ${ }_{\text {KL }} 87_{\text {ac }}$ | 7,183 | 153 TGTCCGGAATCGCAGCTG |
|  |  |  |
|  |  | ATAGGGGGCACTGTGTGACGCGCTCCATGTGCGGGTCGGCAACGT |
|  |  | tTTTCCCGTTTTTAAATATGTCTTCGTCATTITAGATGCAAATATTT |
|  |  | gTCTATGGTCGTTAGACTGCTtTTCCTGCAGGATAGGAAGAAAAA |
|  |  | tattgctttgcgtanacaitanatacctgcgtgggittanaanttt |
| OlaC ${ }_{\text {kL }} 92$ | 5,615 | 602 CCTCTACTGTTATCAGATTCATAAA |
|  |  | CCACAGCTCTGCACTAAAACACAGTCAGTGGTTTATGTGGCGAAT |
|  |  | gCagttittaiancctetgcteccctanctgcagcttccagagag |
|  |  | CATTCAAACCCTACGAGTCTCCAGTCTGTCTGCACACTTCATTAAA |
|  |  | anctttttteattitttattitanctganclatccagctitattaca |
|  |  | CCTGGGGAGGGTAAACGCGAAAAGGCTATTAGGTTAAAGGGTTTG |
| OlaC ${ }_{\text {KL }} 94$ | 4,195 | 646 attaanagcgcatcgitaccattitta |
|  |  | TGTGCATCTCTACTCTTATCAATTCCATCGATTCACAATCTGAGCA |
|  |  | tgittgctgcctttaatancttccaancaggitgcatgiccgatat |
|  |  | tacattittcatccgaggctittctcattagcttccagagtggcag |
|  |  | GAAAAAAGGTTACGGCGCGGTCAGTATGGTAATGGCAGTCAGGA |
|  |  | GAAATGCTTGGGAACAAAAGAAACTTATTTTTCATCTGCGCTGTC |
| OlaC ${ }_{\text {kL }} 95$ | 3,256 | 311 acGCatccctittgiatccgccgigtt |
|  |  | tatattitanaanctatcagittgggittanacgcgantctigiga |
| OlaC $\mathrm{KL}^{9} 96$ | 2,857 | 51 TTTTT |
|  |  |  |
|  |  | tTAGTTGCCTATATGTACCCTGTAGAACCGAATTTGTGTGGAGTAC aAGCATTCGCAAATACGTCTCTACAGGAATACATGGGGAACTGA |
|  |  | AATACCACGCCAACAAAGGATGGCTCTGATTGTTCTTCATCAGCC |
|  |  | ССТССTСАСТСGССТСТАGСTGTTTTССТTСGTССССТССТTССТСТ |
|  |  | gCatacaigcatcagtancgatgcgigcagctcacacclaggia |
| OlaC ${ }_{\text {KL }} 97$ | 2,769 | 551 CCAGCAGTGCTATCTCTTTCCCCTGCAAA |
|  |  | acattataactagttattganctaggigcacgatctganagccat |
|  |  | tTGTGGGGAAAAGAATTCATTGCTGTCTCTCCATCAATAATCCTTG |
|  |  | gCagtganctattgGanccagtcaancgcgagggetganacgcg |
| OlaC ${ }_{\text {kı }} 99_{\text {ab }}$ | 3,711 | 162 GgTCAGGCTGTCTAACTAATATTAAAA |
|  |  | GGTTCCTTATCCGGGAACTACCTCTTTCTCTGCTATTGGGCCATTG |
|  |  | gGtcacgiggttanagtanctitacagggctgctcgcangtagga |
|  |  | gGGctitatgangcagaianacgacanagctaganaiattattt |
| OlaCkı 102 | 151 | 151 CCactccagaiatta |
|  |  | ttattrataggacaattctacattitgitticgtacctatcttgtgc |
|  |  | gGtttcctttangtclanagttatatanaitgcatgitatatgcia |
|  |  | tggattactgcgaataccgatgaattatttitacgtgacacagggt |
|  |  |  |
|  |  | TTTGAATGAAGGTGGAAAAACAGTAAAAAAAAAAAAAAGACATT |
| OlaCLm 103 | 7,707 | 301 ttatcaaattgitattgtgggceat |
|  |  | gttgtgittagaccgicanaigcgantattgtganatgcantan |
|  |  | acgGactttagtgiattegigctantcatattggtcaataanacag |
| OlaCLm 104 | 7,229 | 133 TGAGTGTCTACTAGTtTTAAACACGTTTTGTCGGAGTTTATT |
|  |  | tTATGTTGCTGGACAGATTGGAAAAATTAATGTGCTCGCCACAGT |
|  |  | CGCAGCATCCCTTTAGCCCCCTTTCTAGGGACTGTCGCCTCCATGT |
|  |  | CGCGCAGCTGAAAAGGTAGTTCATTGAAGTTTGGGGGCGAAATGG |
|  |  | Gaganggccgagttcacticgaggacacatttictgtcccattag |
|  |  | GTTCATTTCAGTCTGGATGGCCGACCTCTCCAACCCTGTGACCTGA |
| OlaC $\mathrm{Lm}^{105}$ | 5,763 | 398 CTCACTCTCTATTCGTGTACCTTCTGTG |



ССTTGTCTCCCCCACCCCAGCCACACAACTGGCAGAGACAGTCTG GAATCCAAAGTCACTTTCATTCAGCTGGGTGAAAGAACGTTTTCCC CGAGAACAGCCCGAATTCACCCGAAAACCCTATACACATAAAAC TATGTCCAGGAACTGGATCTTCATCGTGGGGGAGCTGCGTTAGTGC
Cactgacaggatgagacacganccaaaat
Tattancggcacattiatgancaatcaantggccetcgitaanat TATTGACGGGCATAAAATCACGAAGGCCCACAAGCTGAAAAAAA 11 GCTCCATGATTTGGGCTGCTI
gCGCATTGATCCACTGTACAGTTITTGCAGGAGTAAATATAATCAC gTGTCACAGAGGCAGCCAATAGCAGCCGCGAAAGCTCCTAGAAAT

172 CaCATACAGCCCATATAATAATCCGATGCATGTAAA
taitancggcacattta gaacaatcaantggccctcgttanaa 64 ttattgacgggcataaaa
29 GaCaGagatggattacaccatcaantag
18 tTTATGAACAATCAAATG
13 ttgttitattigt
aAATTGCCTTTTTGTGGTGCCAAACAATACGGACAGGATGCTGTGA TTTAGAAGCTAAATCCAGCGCGTTTCCGCTCTGCTGGGACAAAAAA

211 TAAATGACTTTATGGTtTTAATGGACG
Chagatgacgetgitgctccacgin gaggegaghacceaagg tgctgctgcagctetgrggcttggctgaanantgaigancanca 9 AACAGGCCACAG

27 acattitittanttcgianattganat
 aAAACCTTACTGTGTATTTTCTGCAGCCTTGGAGCTGCGTGAGTGT ATAGTGCGCGATGTCATTGCAGCAAACCATCAGATGTTAAATTTTA CGAACTCCTGCGTCTCTGAGATTTACTGCGTTTCCACATCGGTIT 24 CGGGGTCACTATAAATGGGAACCCTGTATTCACACGTCGT

TTATGAATAATACAGCTCCTCTCGAGGTGCACATGCGTAGGCCCCA TACGTCTGAGCGCTCTCTGAAAGATGAGGGGTATGGGTAGGGGGI AAGGAAAAAGAAAGAAAAGAGCAGGGTCTGTGACTGTGCCGGTC GAGCAAGCCCAGCTAACGCGCAAAAAAAAAAAGCACATGCCAT 3 agccgGgittgagcctgcacaangctcggag
tITTATGGTCCTTTTTATGGCTATATGGCTGAAAGGGAGGAAAGAA AGAGTGGCCCCATAAACAGATGAAGAGTCACAGGGAGTCAGAGC 167

TCAGACCCTGTTTTTTTTTCTATAGGATGACCAGGGAAATGCAATC GTTGAAATCCTTTGATGCTACCTAAACCTGGGGAAATIGCAATAGA gattcctgagcgaggagatticagatgttitanattttaangtgic 146 ctatatca

GTGTGTCTGTATTTTTACCACACGGTTGCTATTACCAATAAAGTTGG AAGTACCTGCTGTAAACTTTATTGGCGGTGTTGTATATAACCAGCA 174 CaAAGCACCTCTCCCCCAGCCCTAAAAACTAGTGT

ACATTCCTGGTTGTTAAAGGAGAAATTTACAGCTGAGTAATAAAA 129 GCGCACGTTCTATGAACATGAACTTTATGCTGTTGTCT
tgacctgacctacagcccatagceacaitgigcgcagccatig tTCTGCACTTTTTGGATGGTGTGGCAGCACCTGTGTGCTCCACTGCA GCTCCAGACAGCATTAGCCCAGCATTTCGGAGCCGCTCTGCCAAI cgGttgcantanatttactactgataatanagtttgtcatcagtc - caggcctctgaccagtcagtgacaahttaca

34 tttattiganttgtaagcaaaaaaaaaancgttti
TAAATCCTTGTTTTAAATGCGAGGAGAAAACCGCGGTCTATTGTIT
TGCCCTGAAGTGTTTTAAAAGGCAGATAGCTCTGCATTAAACTCCA TAAAGTCATGAATCTGTCCGCATCACCACATTTAGAACCTCTCTTA CAAATCCCGTCAGGACCAAAAAAATTTTGCCCACTAATGGTTCTA tgagtgcttancgagcatanagtcgi
ataiancactanaactcangggganacantgacctitctantict GCACGCTGGCTTAGCGTCGGCTGCATTCCAGCCTCTCCTGTCTGCT CTTTTCGITTTCAGAGGAGGCGACAAATAGTTTAGACAAATCTCTG CCTCAAATATTATTATTCTTTAACTTGCTGTTCAAGACTGTTTTTAG
262 GCTTTTGAATTATGTGGGGTTTT

GCTTTCATTTCTGAAATGTGCGAGATTTTTTTTTTTTTTTTTTTTTTTTGG CTACAGAAATATTTGCTGGTAAACTGCGGAACTTGTTTTATTGCCC TCGCAGAAGAGACAACATAAAGGCATTCTTACTACCTACACCCTC 210 AATTTATACTCAGTCGAAACATTAA
gTtaAcattctacatcgcattcctccgcgantaccacatttrttaa ACTTTAGTGGAAAAAACCCCGTCTCGTCCAAGCGAGCAGTGCGCC
ACTCCAACTCTGCGTGGTGTTTATCGGCATTTAGCTGTAATGTTCAA GCTCCAAGCTAAAAGCTACAGTTCCCTCTCCACTCCTGССТСТСАА tTGGTGGAGAGTAGTCAGCTGACATTGTCATATTGTCTCTCACCGA
471 GCCAAGCCTCAGCTATAACGCTCG
TAACTACCTAAAAACGAATTACAGACTTGAAAGCGACTCACTGCA tGatgGctaccccgcaactitatcacttatattccacgitaaccac AGGAACGTTCCAAAATATTTCAGGAGCCACTATGGCCACCGTGAC

TGACGCTCTTGTCTTACTGTCCTGCCCTTTTTTGCGCTAAAGCTTCT GCCTGGTCCTCACCAAAGTTTACCGGACACGTTTCTCCTATTCATC CGCACGTGATCTCAAACAAATACCCCATATTTGGGCAGCGCCCAT CGGATCAAGAATTTTTAAAAAAGACAGCAAAGCCTACCCCACCTA 237 TaAATCC

| TniC $\mathrm{k}_{1} 84$ |  | GCAGGAGTGCTGCCTGACCTCCGGATGAACCGCCGGGCAGCAATG ACATCATGACACCGCTCTGTGCGCTICATCACCAATTATGTGTCAC TCTCTGCAGTTTGGAACCTAATGTATTGAATATCACTCACTTTGATC TATTGGTTTACATTTCTAAATGAATTTGCCGCTGTGCGATGATATCC |
| :---: | :---: | :---: |
|  | 7,689 | 217 TTACTTTGCGACGTGCAGAGGACACGAGGTTG |
|  |  | aAtgTgitanatgittagtgangiantacttrtatgittgiagttgi Catttictggianatcctctgactgtatatggtantattgtantgcg CAAATAACTCATtTCCACCTTCCAGAAATGACTTGATTGTTGATTT |
| TniC $\mathrm{KL}^{1} 85$ | 7,250 | 154 ganagattanagtt |
| TniC $\mathrm{K}_{1} 86$ |  | GACAAACGCTTTCTGGAGTTTAAGACCCTCATAAAACTTTATTGCC cCGTtTCCACAGCGCGCTAGTTTCCTGTTTGGTCTGGGAAGGAAGC |
|  | 7,031 | 126 GaCCCAACCTGTGACAACGCTCACCCACCAAAAC |
|  |  | agGitacatatgccaattgccetaagcagggcctgtgantggtgc atagGangcacgiggtgicattrangiggattitatggcctggaig agctgacaanccttcgatatatacacatcatatatantcttanctg |
| TniC кı $^{\text {8 }} 7_{\text {ac }}$ | 6,559 | 153 tccggattcgcagctg |
|  |  | AtagGGggcgatgtgigacacactgitggitggaganaictaiag |
|  |  | CaAaAtGTGTTTATTTTATtTTAAGTATTGATtTTGTAGGCTtTCTTT |
|  |  | tacatatatatcattttattcactgtcattactgcgianattgctgi |
|  |  | GAAGACCTCCTGAAAGAATGCAATGGGTTTGGTtTTACTGTAAAAG |
|  |  | taAataanttcagatgacanttcagantagggccattacattitca |
| TniC ${ }_{\text {kL }} 92$ | 5,286 | 510 gancgatantanttitanganai |
| TniC $\mathrm{K}^{\text {¢ }}$ 93 | 2,948 | 16 anagaaacttattitt |
|  |  | CCACAGCATCGTTAAGACCAACTTGGTCTTTGCATGACAGCAAGTT |
|  |  | TGCTAAAACCGAGTCCTCTGTCTCCCTGCTGCTTTAAACTGTCCAT |
|  |  | gCCCAATTAGTCTCCAGTCTGTCAGCATTTCATTAATACCATTTTTT |
|  |  | tttctagcgaacgitatccaccttanctitattacacctgggaaga |
|  |  | tttaccagganacggctattangttancgctttgattanacgtgcg |
| TniC $\mathrm{KLL}^{94}$ | 4,022 | 663 CCGAAGCAGTTTCCATGGAGCAGA |
|  |  | tGTGCACATCTGCAATGTAACCAATTTCAATGAATCACAAATCCA |
|  |  | agcgtcittggtgitttigatancctcgaancaggtcacatgittg |
|  |  | atactgcattiticattctggcettticteattagctictctagtgac |
|  |  | AGGAAGAAAGGTTACGGGGCGgTCAGTATGGTAATAGCAGACAG |
|  |  | Aagaiatggctggaaicaianganactiattittcatcagtcctg |
| TniC $\mathrm{KLL}^{95}$ | 3,149 | 312 TCACGCCATCCCTTTGTACCCTGTGTG |
|  |  | ttagttgtctatatgtaccetgtaganccgantttgtgtaagttcag |
|  |  | acagtcacangtacgtctctacaggantacatggacaictganaa |
|  |  | TCCACAGCGGCCACTGGACACTCCTGCTTCTCCTCCTCTGCACACC |
|  |  | тTTCС TССТTTTTCACTCCAAGCATCTGTGTTTTACACTCAGTCTGC atcCagtcgctgiagcatantctigtcacaiagigiattcatgatc |
| Tnic $\mathrm{KL}^{9} 9$ | 2,679 | 492 Cagacagtcgaggcttgigtgtag |
| TniC $\mathrm{KLL}^{98}$ | 1,114 | 31 attanaaccctattangatcceatticattc |
|  |  | acattatanctagttattganctaggtgcgcgatctaanagccatt tGTGTGGATAAGGAATTCATTGCTGTCCCTCCATCAATAACCCTTG |
|  | 3,522 | GCAGTGAACTATTGGAACCAGTCAAACGCGAGGGGTGAAACGCG 163 GGTCAGCCTGTCTAACTAATATTAAAA |
| TniC $\mathrm{KL}^{\text {¢ }} 99_{\text {ab }}$ |  | GGTTCCTTATCCGGGAACTACCTCTAAGCCCACTATTGGCCCATTG tgTCACGTGGTAAAAGTAACTTTACAGGGCTGCTCGCAAGTAGGA gGGCTTTATGGAGCAGAAAAACGACAAAGCTAGAAAAATTATTTT |


| Danio rerio zebrafish Hox Cb |  |  |
| :---: | :---: | :---: |
| PFC | Dist to 3' gene | Length Sequence |
| DreCB ${ }_{\text {DE }} 23$ | 1,295 | 21 agaangcagttitaanaataa CTCCTACGTTTGCAACCGTTCCAAATTGATATATGAGAATATCTACT |
| DreCB ${ }_{\text {DE }} 26_{\text {b }}$ | 168 | 59 ttcgatcacgtg |
| DreCB ${ }_{\text {EJ }} 32$ | 14,468 | 20 gGaaaattangcctitttat |
| DreCB ${ }_{\text {EJ }} 47$ | 12,556 | 17 TCTCTGGAGCTCATtTC |
| DreCB ${ }_{\text {EJ }} 54$ | 12,259 | 43 TGTTATGATTATGTACACCATGGGGATATGTGGTGACGTCACT <br> CAGCTGATGCGTGGTTTAGGTAGTTTGATGTTGTTGGGGTTGACTTC |
| DreCB ${ }_{\text {FG }} 40_{\text {abc }}$ | 11,859 | 72 CtGGctcgacaicangaiactgcct |
| DreCB ${ }_{\text {EJ }} 76$ | 7,169 | 32 tattanatctacagtceattgagggcattgan |
| $\mathrm{DreCB}_{\mathrm{EJ}} 67_{\mathrm{b}}$ | 3,950 | 30 gaaantttacagctatgtantanaigitta |


| PFC Hornshark D | Frog D | Coelacanth D | Human D | Bichir D | Zebrafish D | Medaka Da | Tetraodon Da | Medaka Db | Tetradodon Db |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| $1 \mathrm{HfrD}_{\mathrm{Ac}} \mathrm{l}_{\mathrm{ab}}$ | $\mathrm{XtrD}_{\text {ac }} \mathrm{l}_{\text {ab }}$ | $\mathrm{LmeD}_{\text {AD }} 1_{\text {ab }}$ | $\mathrm{HsaD}_{\mathrm{Ac}} \mathrm{l}_{\mathrm{ab}}$ |  | DreD $\mathrm{Ac} \mathrm{l}_{\mathrm{b}}$ | $\mathrm{OlaDA}_{\text {AD }} 1_{\mathrm{b}}$ | $\mathrm{TniDA}_{\text {AD }} 1_{\mathrm{b}}$ |  |  |
| 2 |  |  |  |  | DreD ${ }_{\text {cD }}{ }^{2}$ | $\mathrm{OlaDA}_{\text {AD }}{ }^{2}$ |  |  |  |
| 3 |  |  | $\mathrm{HsaD}_{\text {AC }} 3$ |  |  | $\mathrm{OlaDA}_{\text {AD }} 3$ |  | ） | － |
| 4 |  |  |  | 老 | DreD ${ }_{\text {cD }} 4$ |  | TniDA ${ }_{\text {AD }}{ }^{4}$ |  |  |
| 5 － | XtrD ${ }_{\text {CE }} 5$ |  | $\mathrm{HsaD}_{\mathrm{cD}} 5$ |  |  |  |  |  |  |
| 6 | $\mathrm{XtrD}_{\text {CE }} 6$ |  | $\mathrm{HsaD}_{\mathrm{CD}} 6$ |  |  |  |  |  |  |
| 7 |  |  |  |  | DreD ${ }_{\text {DE }} 7$ |  | TniDA ${ }_{\text {DE }} 7$ |  |  |
| 8 |  |  | $\mathrm{HsaD}_{\text {DE }} 8$ |  |  | OlaDA ${ }_{\text {DE }} 8$ |  |  |  |
| $9 \mathrm{HfrD}_{\text {DE }} 9_{\text {abcd }}$ |  | $\mathrm{LmeD}_{\text {DE }} 9_{\text {bcd }}$ |  |  | DreD $\mathrm{DE}_{\text {de }} 9$ abcde | $\mathrm{OlaDA}_{\text {dE }} 9_{\text {abce }}$ | TniDA ${ }_{\text {DE }} 9_{\text {abe }}$ |  |  |
| $10 \mathrm{HfrD}_{\text {dE }} 10_{\text {bode }}$ | $\mathrm{XtrD} \mathrm{CEF}^{1} 0_{\text {abcde }}$ | $\mathrm{LmeD}_{\text {DE }} 10_{\text {bcde }}$ | $\mathrm{HsaD}_{\text {DE }} 100_{\text {abcde }}$ |  | DreD $\mathrm{DE} 10_{\text {cd }}$ | $\mathrm{OlaDA}_{\text {DE }} 10_{\text {d }}$ | $\mathrm{TniDA}_{\text {DE }} 10_{\text {cd }}$ |  |  |
| $11 \mathrm{HfrD}_{\mathrm{EF}} 11_{\mathrm{ab}}$ | $\mathrm{XtrD}_{\text {EF }} 11_{\text {ab }}$ | $\mathrm{LmeD}_{\mathrm{EF}} 11_{\mathrm{ab}}$ | $\mathrm{HsaD}_{\mathrm{EF}} 11_{\mathrm{ab}}$ |  | DreD ${ }_{\text {EF }} 1^{1}$ | $\mathrm{OlaDA}_{\text {EF }} 1_{\text {ab }}$ | $\mathrm{TniDA}_{\text {EF }} 11_{\text {ab }}$ |  |  |
| $12 \mathrm{HfrD} \mathrm{EF}^{12}$ |  |  | $\mathrm{HsaD}_{\mathrm{EF}} 12$ |  |  |  |  |  | ， |
| 13 |  |  | $\mathrm{HsaD}_{\text {EF }} 13$ |  | DreD EFF 13 |  |  | － | － |
| 14 | $\mathrm{XtrD}_{\text {EF }} 14$ |  | $\mathrm{HsaD}_{\text {EF }} 14$ |  |  |  |  |  |  |
| 15 | $\mathrm{XtrD}_{\text {EF }} 15$ |  | $\mathrm{HsaD}_{\text {EF }} 15$ | 烼 |  |  |  |  |  |
| 16 |  |  | $\mathrm{HsaD}_{\text {EF }} 16$ | 絓 | DreD ${ }_{\text {EF }} 16$ |  |  |  | ， |
| 17 |  |  | $\mathrm{HsaD}_{\text {EF }} 17$ |  |  | $\mathrm{OlaDA}_{\text {EF }} 17$ |  | $\left.\square^{\square}\right)^{\text {a }}$ |  |
| 18 | $\mathrm{XtrD}_{\text {EF }} 18$ |  | $\mathrm{HsaD}_{\text {EF }} 18$ |  |  |  |  |  |  |
| $19 \mathrm{HfrD}_{\mathrm{EF}} 19_{\mathrm{ac}}$ | $\mathrm{Xtr} \mathrm{DEF} 19_{\text {bc }}$ | $\mathrm{LmeD}_{\mathrm{EFF}} 19 \mathrm{abc}$ | $\mathrm{HsaD}_{\mathrm{EF}} 19_{\text {abc }}$ |  | DreD $\mathrm{EFF}^{19} 9$ | $\mathrm{OlaDA}_{\text {EF }} 199_{\text {ac }}$ | TniDA ${ }_{\text {EF }} 19{ }_{\text {ac }}$ |  |  |
| 20 |  |  | $\mathrm{HsaD}_{\mathrm{FG}} 20$ |  |  | $\mathrm{OlaDA}_{\text {FG }} 20$ |  |  |  |
| $21{ }^{\text {HfrD }}{ }_{\text {FG }} 21_{\text {abc }}$ | $\mathrm{XtrD}_{\mathrm{FG}} 21_{\mathrm{b}}$ | $\mathrm{LmeD}_{\mathrm{FG}} 211_{\text {ab }}$ | $\mathrm{HsaD}_{\mathrm{FG}} 21_{\mathrm{ab}}$ |  | DreD $\mathrm{FGG}^{2} 1_{\text {abc }}$ |  | TniDA ${ }_{\text {FG }} 21_{\text {abc }}$ | $)^{\text {a }}$ O ${ }^{\text {a }}$ |  |
| 22 |  |  |  |  | $\mathrm{DreD}_{\mathrm{FG}} 22$ | $\mathrm{OlaDA}_{\mathrm{FG}} 22$ | $\mathrm{TniDA}_{\mathrm{FG}} 22$ | \％ | （ |
| $23 \mathrm{HfrD}_{\mathrm{FG}} 23_{\text {abce }}$ | $\mathrm{XtrD}_{\mathrm{FG}} 23_{\text {abce }}$ | $\mathrm{LmeD}_{\mathrm{FG}} 23_{\text {abce }}$ | $\mathrm{HsaD}_{\mathrm{FG}} 23_{\text {abe }}$ |  | DreD ${ }_{\mathrm{FG}} 23_{\mathrm{bcd}}$ | $\mathrm{OlaDA}_{\text {FG }} 23_{\text {bcd }}$ | TniDA $_{\text {FG }} 23_{\text {bcd }}$ |  |  |
| 24 |  |  | $\mathrm{HsaD}_{\mathrm{GH}} 24$ |  |  |  |  |  | TniDB ${ }_{\text {GL }} 24$ |
| 25 |  | $\mathrm{LmeD}_{\text {GH }} 25$ | $\mathrm{HsaD}_{\mathrm{GH}} 25$ |  |  |  |  |  |  |
| 26 |  |  | $\mathrm{HsaD}_{\text {GH }} 26$ |  |  |  |  |  | TniDB ${ }_{\text {GL }} 26$ |
| 27 |  |  | $\mathrm{HsaD}_{\mathrm{GH}} 27$ |  |  | $\mathrm{OlaDA}_{\text {GL }} 27$ |  |  |  |
| 28 |  | $\mathrm{LmeD}_{\text {GH }} 28$ | $\mathrm{HsaD}_{\mathrm{GH}} 28$ |  |  |  |  |  |  |
| 29 |  |  |  | 巟 | $\mathrm{DreD}_{\mathrm{GL}} 29$ | $\mathrm{OlaDA}_{\text {GL }} 29$ |  |  |  |
| 30 |  |  | $\mathrm{HsaD}_{\mathrm{HL}} 30$ | 立 | DreD $\mathrm{CLL}^{30}$ |  |  |  |  |
| 31 |  |  | $\mathrm{HsaD}_{\mathrm{HL}} 31$ |  | DreD ${ }_{\text {GL }} 31$ |  |  |  |  |
| 32 |  |  |  |  |  | $\mathrm{OlaDA}_{\text {GL }} 32$ | $\mathrm{TniDA}_{\text {cL }} 32$ |  |  |
| 33 | $\mathrm{XtrD}_{\text {HL }} 33$ |  | $\mathrm{HsaD}_{\mathrm{HL}} 33$ |  |  |  |  |  |  |
| $34 \mathrm{HfrD}_{\mathrm{HK}} 34$ |  |  | $\mathrm{HsaD}_{\mathrm{HL}} 34$ |  |  |  |  |  |  |
| $35 \mathrm{HfrD}_{\mathrm{HK}} 35_{\text {abc }}$ | $\mathrm{XtrD}_{\mathrm{HL}} 35_{\text {abc }}$ | $\mathrm{LmeD}_{\mathrm{HL}} 35_{\text {ab }}$ | $\mathrm{HsaD}_{\mathrm{HL}} 35_{\text {abcd }}$ |  | DreD $\mathrm{GLL} 35_{\text {bed }}$ | OlaDA ${ }_{\text {cL }} 35_{\text {bcd }}$ | $\mathrm{TniDA}_{\text {GL }} 35_{\text {bc }}$ |  |  |
| 36 |  |  | $\mathrm{HsaD}_{\mathrm{HL}} 36$ |  | $\mathrm{DreD}_{\mathrm{GL}} 36$ |  |  |  |  |
| $37 \mathrm{HfrD}_{\text {kL }} 37_{\mathrm{bc}}$ | XtrD ${ }_{\text {HL }} 37_{\text {bc }}$ | $\mathrm{LmeD}_{\text {HL }} 37_{\mathrm{b}}$ | $\mathrm{HsaD}_{\mathrm{HL}} 37_{\mathrm{bc}}$ |  | DreD $\mathrm{ciL} 37_{\text {abcd }}$ | OlaDA ${ }_{\text {GL }} 37_{\text {abcd }}$ | TniDA $_{\text {GL }} 37_{\text {abcd }}$ |  |  |
|  |  |  |  |  | DreD CLL 38 | $\mathrm{OlaDA}_{\text {GL }} 38$ | $\mathrm{TniDA}_{\text {GL }} 38$ |  |  |
|  |  |  | $\mathrm{HsaD}_{\mathrm{HL}} 39$ |  |  |  |  |  | $\mathrm{TniDB}_{\text {GL }} 39$ |
|  | XtrD ${ }_{\text {HL }} 40_{\mathrm{a}}$ |  | $\mathrm{HsaD}_{\mathrm{HL}} 40_{\mathrm{ab}}$ |  | DreD ${ }_{\text {GL }} 40_{\text {ab }}$ | $\mathrm{OlaDA}_{\text {GL }} 40{ }_{\text {ab }}$ | TniDA ${ }_{\text {GL }} 40_{\text {ab }}$ |  |  |
| 41 者 |  |  | $\mathrm{HsaD}_{\mathrm{HL}} 41$ |  |  |  |  |  | TniDB ${ }_{\text {GL }} 41$ |
|  |  |  | $\mathrm{HsaD}_{\mathrm{HL}} 42$ |  |  |  |  | $\mathrm{OlaDB}_{\text {GL }} 42$ |  |
|  |  |  |  |  |  |  |  | $\mathrm{OlaDB}_{\mathrm{GL}} 43$ | TniDB ${ }_{\text {GL }} 43$ |
|  |  |  |  |  |  |  |  | $\mathrm{OlaDB}_{\mathrm{GL}} 44$ | TniDB ${ }_{\text {GL }} 44$ |
|  |  |  |  |  |  |  |  | $\mathrm{OlaDB}_{\text {GL }} 45$ | $\mathrm{TniDB}_{\text {GL }} 45$ |
|  | XtrD ${ }_{\text {HL }} 46_{\text {abcd }}$ | $\mathrm{LmeD}_{\mathrm{HL}} 46_{\text {abcde }}$ | $\mathrm{HsaD}_{\text {HL }} 46_{\text {abcde }}$ |  | DreD ${ }_{\text {GL }} 46_{\text {bed }}$ | $\mathrm{OlaDA}_{\text {GL }} 46_{\text {cde }}$ | TniDA ${ }_{\text {GL }} 46_{\text {b }}$ |  |  |
|  | $\mathrm{XtrD}_{\mathrm{HL}} 47_{\text {abc }}$ | $\mathrm{LmeD}_{\mathrm{HL}} 47_{\text {abc }}$ | $\mathrm{HsaD}_{\mathrm{HL}} 47_{\text {abc }}$ |  | $\mathrm{DreD}_{\mathrm{GL}} 47_{\mathrm{b}}$ |  |  |  |  |
|  | XtrD ${ }^{4 L} 48_{\text {abcde }}$ | $\mathrm{LmeD}_{\mathrm{HL}} 48_{\text {abcde }}$ | $\mathrm{HsaD}_{\text {HL }} 48_{\text {abcale }}$ |  | DreD $\mathrm{CLL} 48_{\text {abcde }}$ | $\mathrm{OlaDA}_{\text {GL }} 48_{\text {abcale }}$ | $\mathrm{TniDA}_{\text {GL }} 48_{\text {abcd }}$ | $\mathrm{OlaDB}_{\text {GL }} 48_{\text {bc }}$ | $\mathrm{TniDB}_{\text {GL }} 48_{\text {ab }}$ |
|  | $\mathrm{XtrD}_{\mathrm{HL}} 49_{\mathrm{abc}}$ | $\mathrm{LmeD}_{\mathrm{HL}} 49_{\text {abc }}$ | $\mathrm{HsaD}_{\mathrm{HL}} 49_{\text {abc }}$ |  | $\mathrm{DreD}_{\mathrm{GL}} 49_{\mathrm{b}}$ | $\mathrm{OlaDA}_{\text {GL }} 49{ }_{\text {bc }}$ | TniDA ${ }_{\text {GL }} 49_{\mathrm{b}}$ |  |  |
| 50 将 |  |  |  | $\mathrm{PseD}_{\text {LM }} 50$ | $\mathrm{DreD}_{\mathrm{LM}} 50$ |  |  | \％${ }_{\text {－}}$ |  |
| 51 v／mumbumb |  |  |  | $\mathrm{PseD}_{\text {LM }} 51$ | $\mathrm{DreD}_{\text {LM }} 51$ |  |  |  |  |
|  |  |  |  |  |  | $\mathrm{OlaDA}_{\text {LM }} 52$ | $\mathrm{TniDA}_{\text {LM }} 52$ |  |  |
| 53－mmumumun |  |  |  |  |  | $\mathrm{OlaDA}_{\text {LM }} 53$ | $\mathrm{TniDA}_{\text {LM }} 53$ | Hex ${ }^{\text {a }}$ |  |
| 54， |  |  |  | $\mathrm{PseD}_{\text {LM }} 54$ |  | $\mathrm{OlaDA}_{\text {LM }} 54$ |  |  |  |
|  |  |  |  |  |  | $\mathrm{OlaDA}_{\text {LM }} 55$ | $\mathrm{TniDA}_{\text {LM }} 55$ | H | Hele |
|  | $\mathrm{XtrD}_{\mathrm{LM}} 56_{\text {abccle }}$ | $\mathrm{LmeD}_{\text {LM }} 56_{\text {abcde }}$ | $\mathrm{HsaD}_{\mathrm{LM}} 56_{\text {abcde }}$ | $\mathrm{PseD}_{\text {LM }} 56_{\text {bcd }}$ | $\mathrm{DreD}_{\text {LM }} 56{ }_{\mathrm{c}}$ |  |  |  |  |
|  | $\mathrm{XtrD}_{\mathrm{LM} 5} 5$ |  | $\mathrm{HsaD}_{\text {LM }} 57$ |  |  |  |  | $)^{\text {P }}$ |  |
| 58 － | $\mathrm{XtrD}_{\mathrm{LM}} 58_{\text {bc }}$ | $\mathrm{LmeD}_{\text {LM }} 58_{\text {bcd }}$ | $\mathrm{HsaD}_{\mathrm{LM}} 58_{\text {bd }}$ | $\mathrm{PseD}_{\text {LM }} 58_{\text {bcd }}$ | DreD ${ }_{\text {LM }} 58_{\text {abcd }}$ | $\mathrm{OlaDA}_{\text {LM }} 58_{\text {abcd }}$ | $\mathrm{TniDA}_{\text {LM }} 58_{\text {abcd }}$ |  |  |
|  |  |  |  |  | $\mathrm{DreD}_{\mathrm{LM}} 59$ | $\mathrm{OlaDA}_{\text {LM }} 59$ |  |  | He |
|  |  |  |  |  | $\mathrm{DreD}_{\text {LM }} 60$ | $\mathrm{OlaDA}_{\text {LM }} 60$ | TniDA ${ }_{\text {LM }} 60$ |  |  |
|  | XtrD ${ }_{\text {LM }} 61_{\text {ab }}$ | $\mathrm{LmeD}_{\text {LM }} 61{ }_{\text {ab }}$ | $\mathrm{HsaD}_{\text {LM }} 61_{\text {abc }}$ | $\mathrm{PseD}_{\text {LM }} 611_{\text {ab }}$ | DreD ${ }_{\text {LM }} 61_{\text {abcd }}$ |  | TniDA ${ }_{\text {LM }} 61{ }_{\text {acd }}$ |  | － |
|  | $\mathrm{XtrD}_{\mathrm{LM}} 62_{\mathrm{a}}$ | $\mathrm{LmeD}_{\text {LM }} 62_{\text {ab }}$ | $\mathrm{HsaD}_{\mathrm{LM}} 62_{\text {ab }}$ | $\mathrm{PseD}_{\mathrm{LM}} 62_{\text {ab }}$ | DreD ${ }_{\text {LM }} 62_{\text {ab }}$ | OlaDA ${ }_{\text {LM }} 62_{\mathrm{a}}$ |  |  |  |
|  |  |  |  | $\mathrm{PseD}_{\text {LM }} 63$ |  | $\mathrm{OlaDA}_{\text {LM }} 63$ |  | O |  |
|  |  | $\mathrm{LmeD}_{\text {LM }} 64$ | $\mathrm{HsaD}_{\mathrm{LM}} 64$ |  |  |  |  |  |  |
|  |  | $\mathrm{LmeD}_{\text {LM }} 65$ | $\mathrm{HsaD}_{\text {LM }} 65$ |  |  |  |  |  | － |
|  |  |  | $\mathrm{HsaD}_{\text {LM }} 66$ |  |  |  | TniDA ${ }_{\text {LM }} 66$ |  |  |
|  | $\mathrm{XtrD} \mathrm{LM}^{67} 7_{\text {ab }}$ | $\mathrm{LmeD}_{\text {LM }} 67{ }_{\text {abc }}$ | $\mathrm{HsaD}_{\text {LM }} 67{ }_{\text {abc }}$ | $\mathrm{PseD}_{\text {LM }} 67{ }_{\text {bc }}$ | DreD $_{\text {LM }} 67_{\text {bc }}$ | $\mathrm{OlaDA}_{\text {LM }} 677_{\text {bc }}$ | $\mathrm{TniDA}_{\text {LM }} 67_{\text {bc }}$ | H $\square_{\square}$ | H ${ }^{\text {H }}$ H |
|  |  | $\mathrm{LmeD}_{\text {LM }} 68$ | $\mathrm{HsaD}_{\text {LM }} 68$ |  |  |  |  |  |  |
|  |  | $\mathrm{LmeD}_{\text {LM }} 699_{\text {abcd }}$ | $\mathrm{HsaD}_{\text {LM }} 699_{\text {abd }}$ | $\mathrm{PseD}_{\text {LM }} 69_{\text {abcd }}$ | DreD ${ }_{\text {LM }} 69_{\text {abcd }}$ | $\mathrm{OlaDA}_{\text {LM }} 69_{\text {bc }}$ | TniDA ${ }_{\text {LM }} 69_{\text {bc }}$ | －${ }^{\text {a }}$ |  |
|  |  | $\mathrm{LmeD}_{\text {LM }} 70_{\text {ab }}$ | $\mathrm{HsaD}_{\mathrm{LM}} 70_{\mathrm{a}}$ | $\mathrm{PseD}_{\mathrm{LM}} 70_{\mathrm{ab}}$ | $\mathrm{DreD}_{\mathrm{LM}} 70_{\text {ab }}$ | $\mathrm{OlaDA}_{\text {LM }} 70_{\text {ab }}$ | $\mathrm{TniDA}_{\text {LM }} 70_{\text {ab }}$ |  |  |
|  |  | $\mathrm{LmeD}_{\text {Mo }} 71$ | $\mathrm{HsaD}_{\mathrm{Mo}} 71$ |  |  |  |  |  |  |
| 72－ |  | $\mathrm{LmeD}_{\text {Mo }} 72$ | $\mathrm{HsaD}_{\mathrm{MO}} 72$ |  |  |  |  |  |  |
|  |  | $\mathrm{LmeD}_{\text {MO }} 73$ | $\mathrm{HsaD}_{\text {MO }} 73$ |  |  |  |  |  | － |
|  |  |  | $\mathrm{HsaD}_{\mathrm{MO}} 74$ | $\mathrm{PseD}_{\text {No }} 74$ |  |  |  |  |  |



| Latimeria menadoensis coelacanth Hox D |  |  |
| :---: | :---: | :---: |
|  | Dist to 3 ' gene | Length Sequence |
|  |  | CtGGTCAAAATGACCCATGCATCCTTTCTAGCCCGAAATGTCAT |
|  |  | tcatcanaiagttgtgctegtcattanggtaggaitgacgetgi |
|  |  | tggantantcatttattgraacagctttataagcanataatac |
|  |  |  |
|  |  | antacatclaggtagagacagantagangtgantactagatct |
| $L E m e D ~_{\text {AD }} 1_{\text {ab }}$ | 59,957 | 331 CTTGTCCTAAGCTTGATCACTGCTTGGCTGGATTGTT |
|  |  |  |
|  |  | ttctctigcctgctatttttacgagagctgicagacagigTctg |
|  |  | ttcatgttctccagatactgggggcgccacancanagttanggt |
| $L_{\text {Lme }} \mathrm{DE}_{\text {DE }} 10_{\text {bcde }}$ | 2,950 | 145 caagttagtgtct |
|  |  | tatggtanttictacattgagacacgigacgiaattactcctag |
| $\mathrm{LmeD}_{\mathrm{DE}} 9^{\text {bcd }}$ | 191 | 70 aftcgatcangatccattacacgica |
| $L_{\text {meD }}^{\text {EF }} 11_{\text {ab }}$ | 5,450 | 28 tgittatanaaccttganctgtctagac |
|  |  | gtcgetgitcaccatattigaggiganagtcaaccatcancanc |
|  |  | Cacgigactectancgaggtagtgictcangcceattichant |
|  |  | ttcattgactictctgtcatgiggatctatagagacactcacaat |
|  |  | tatacagagaatattticctagagatgicagcctacaaiggac |
| $\mathrm{LmeD}_{\text {EF }} 11_{\text {abc }}$ | 207 | 177 A |
| $\mathrm{LmeD}_{\mathrm{FG}} 21_{\mathrm{ab}}$ |  | ttttattaggaagciattatagratantcanatgcacctcatan |
|  | 726 | 46 AA |
|  |  | agcgaittggttactcctcatatcgatanatataaccacgita |
|  |  | gCTCCGTAACCAATGGTTGAAGTCGCTATCTGCAAAATACTATG |
| $\mathrm{LmeD}_{\text {FG }} 23_{\text {abce }}$ | 160 | 130 attGttgagagtggancgiatctittactctiancagtgian |
|  |  | GAAGGTGTTGGTGCTGTATCACTATTTTCACGCGTTTACCTATAA tgCaAttanagcaggaagtancggatacantanacccataga |
|  |  | attggctagacgtctggagctaatgagittatgagttgiaactt |
|  |  |  |
|  |  | ctgctggcgacaigcancgatgcaancacaantattgcang |
| $\mathrm{LmeD}_{\text {GH }} 25$ | 5,468 | 235 anatctagacantana |
| $\mathrm{LmeD}_{\text {GH }} 28$ | 3,362 | 33 AAAATCCATTGGTCTTCCATTAAGGTACAAACA |
|  |  |  |
| Lme $_{\text {Hıl }} 35_{\text {ab }}$ | 19,854 | 52 atttaca |
|  |  | cacgigatcgcantanaactigttttatgacaaggaagttgaca |
| LmeD ${ }_{\text {HL }} 37_{\text {b }}$ | 6,869 | 46 AG |
|  |  | aAtanctctggcttigacctatctgaccangicgcacaitaagg |
| LmeD ${ }_{\text {HL }} 46_{\text {abode }}$ | 2,687 | 81 tganattcaggtcacancgtctancanattiganait |
|  |  | tgaactittgtacticttegcgtgattgicgacanagtanaata |
| LmeD HL $^{4} 77_{\text {abc }}$ | 2,053 | 86 atganacttiglgatatgittgtanatgatteagtatgacc |
|  |  | gtctatatataccctgtaganccganttigtgtaatgitatcag |
| LmeD ${ }_{\text {HL }} 48_{\text {abode }}$ | 1,286 | 82 antcacagattcanttctagg igagtatatggtcgatg |
|  |  | tgataanctccttggattittattghctatctctgicacatggat |
|  |  | acctaactitattcagtigacagcangiaggaggictctatgra |
|  |  | gGGagaganaianaiagacanctcgagananattagtattt |
| LmeD HL $^{4} 9_{\text {abc }}$ | 153 | 147 Ctaccttcagaaatta |
|  |  | attgittgtatgacanaagcacagaattcacgcatagcanag |
|  |  | acgacttctitttaacgicttcacgitgagangctganaaggra |
|  |  | ttitactgangttcgactaanctgcaganacaggttcaactagg |
|  |  | gGacaaatttctattcgattagttgiatttcagccgagagagc |
| $L_{\text {LmeD }}^{\text {L }} 56_{\text {abcde }}$ | 12,504 | 203 TGACCTCTAAACCCTTGACCTTTTGGAC |
|  |  | Cacgigatttactaantaattanttcagtacgicccctangaia |
|  |  | CacgGcgicgicattantcgaccangcanagcactctatcaga |
|  |  | Cttganaictgangagatclcaagaitatantatacaancgat |
|  |  | gCagtatcctanttctactcttgaagctctttggtancattgg |
| LmeD $\mathrm{Lm}^{5} 8_{\text {bcd }}$ | 10,151 | 198 acaantgGangcttggtaggtana |
|  |  | gaacaitgccgactgtcagtccatctgctcctattcttanagac |
|  |  | gGtGAGAAAAGGGCCTGGACTTCTTTTCAATCACGGGTAGTAA |
|  |  | attittcttigcgicattagaganaggctatanaactgagtcga |
| LmeD ${ }_{\text {Lm }} 64$ | 9,650 | 149 ATGTTTCCCAAGGCAGGT |
|  |  | anagtttcctttttagagctacattaggataatanaccattana |
|  |  | ttagaggttcaangatgacatatactticcagtticagtagtga |
|  |  | ttgGgGanataattcattggttgcteatagctgctcatttigtt |
| LmeD ${ }_{\text {Lm }} 65$ | 9,074 | 143 gaccagatc |
|  |  | TGACAATAGCCAGAATTGTTATAAATCATTCTAAGTAATTCATG |
|  |  | AAACAGTGCGaGGCTGTTGGGGGccgggcgangattctanatc |
| $\mathrm{LmeD}_{\text {LM }} 67_{\text {abc }}$ | 8,770 | 116 tttcagttitattgccccatgancatatg |
| $L_{\text {LmeD }}^{\text {Lm }}$ 68 | 8,090 | 21 atattttactgtanaitataa |
|  |  | CTCAATCAGAACAATCTGGTATACAGATCACGGGAACAAATAT |
| $L_{\text {meD }}^{\text {Lm }}$ 69aba ${ }_{\text {abd }}$ |  | 92 GCTCGTATTTTAAGGCAGCGCCTATATTTGTGATTATAAAAGGTT |
|  | 7,246 | 92 TCCG |
|  |  | tttattgatagitgagcctaagactgittccattctatcgagait |
|  |  | aCtGTCTGCACTGGTATATGGAAATGTCTGTAAAACTGCAAGAT |
|  |  | Caggittaggacagtattgiccgcagacaanggglganggata |
|  |  |  |
|  |  | caggtttatgatanattgatatacaggtaagctatagcatgaca |
| LmeD $\mathrm{Lm}^{7} 70 \mathrm{ab}$ | 7,026 | 233 afatgGaicctttaa |
| LmeD $\mathrm{LM}^{6} 61{ }_{\text {ab }}$ | 4,621 | 42 gitcattangGGgtgagttattgctgratangccaangatca |
|  |  | gTCatanattitgtigcaanccccaatgacaggtgcattgatat |
| LmeD ${ }_{\text {LM }} 62_{\text {ab }}$ | 2,302 | 45 G |
|  |  | ttanttiatgractitttcantantgtcleanticcattanacti |
|  |  | gatatgtattattattitanatangagcatttigtatcacttat |
|  |  | ttatcCttgicttantgratteatgtgancatttgtagaictict |
|  |  | acagcatgacgrgaacgagttgattcagatccatggtcatta |
|  |  | atttttcctctttagtanactiggittganatagttccganagta |
| $\mathrm{LmeD}_{\text {мо }} 71$ | 19,338 |  |
| $\mathrm{LmeD}_{\text {M0 }} 72$ | 9,621 | 30 anaathanagaactgagcatgitcacagt |
| $\mathrm{LmeD}_{\text {Mo }} 73$ | 7,631 |  |



CACGTGATTGGCGAAATAATTAATTCAGCACGTCCCTTAAGAA ACACGGAGTCGTCATTAATCTGCCACGCAAAGGGCTCTCTCCG ACTTGGAAAGTGCAGGGATCCCAAGAATATCACCCGTCCAGG 198 CGGGCGCGGGAGCGCGGCCGCAGGTAAA gTTCATTAAGGGGTGAGTTATTGCGGTGCGAGCCAAAGGTCAC 7 tTCAAAGGCTTATG
gTCATAAATTTTGCTACAAACCACAATGACAGGTGCATTGATA 45 TG

GAACAATGGTCGCTGTCACGGCATCTGCCGCCTATTCTTAAAC CGGTGAGAAAAGGCCCTGGCCCTCTTTTCAAGCGAGGGTCGTA 149 aAttTTACCCCAGGCAGGT
aAAGTTTCCCCCATTATGAATTATACATTCAAACAATAACACA TTAATTCAATTATTCAAAGATGACAAATGTTTATGTGCTTTGCG AGTGACTCGGGCGCAGATTCCAGGCGCTTTCTCTGAGCTGCTT 151 GCATTTTTCTCAATGAGAATC
24 CGGCTTGGCGGGCACTGGCCCGGG
TGACAATTGCCCGGGTTGGTGTGATAAATCATCGTAAGTAATT CCTGAAAGGGTGCGAGACTGTTGGGGGCCGGGCGAGGACTGT 119 aAATCTTTCCGGTTTATTGCTCTATGAACATATG
17 atatttctaanatataa
CTCAATCAGTACAAGCTTCCCTCGGGGTCACGTGAACAAATAT gCTTGCATTTGAAGGCAGCGTCTGTATTTCCCGACTATGAGGG

TTTATTGGTAGTTGAACCTCAGCCTGGTTCCGTTCTACCGGGAA TTCCGTGTGCTCGAGTATATGGCCGTGTCTGCGAGCGCGCAAG CTAGCTCTGCCCCCCACTGGCGCCCACTCTGAGGCCGAGGACA 204 CCAGGTTTATGATAAATTGGGATCCAGGTAAG

TTAATCTATATTCTCCTTCCTGTGCCGTAAGGATTGCATCGGAC TAAACTATCTGTATTTATTATTTGAAGCGAGTCATTTCGTTCCCT GATTATTTATCCTTGTCTGAATGTATTTATGTGTATATTTGTAGA TTTATCCAGCCGAGCTTAGGAATTCGCTTCCAGGCCGTGGGGG 491 GAGTAGTTTTGAACAGTCGTAACCGTGGCTGGTG
25 AAAAATAAAAGAAGTCGGGCACAGT
27 aAAAAAAAATTCCTGAGTCAGACTATI
18 AAAACAAAACAAAACAAA




## Table S2-Conserved PFC Data Supplement to Figure 9

Data in this table corresponds to Fig. 9. For each hox cluster the order in this file corresponds to the order in the figure from left to right. Each PFC is also color coordinated with the lines representing them in Fig. 9. The groups are coded as such 1) horn shark, 2) >2 lobe-finned fish, 3) bichir, 4) >2 teleost fish. In this analysis the PFC was color coded according to the follwing code: Red lines represent PFCs that are found within untranslated regions (UTRs) of Hox gene mRNAs. Blue lines represent PFCs that are found within 500 nucleotides of a Hox gene proper region but have not been identified as UTRs. Green lines represent PFCs that have high or identical sequence identity to sequences in the EST library. Yellow lines represent PFCs that are not found in any databases or published papers. Black lines are sequences with known function and labeled accordingly. If a PFC fits into more than one category then colors are used in this decreasing order: Black, Red, Blue, Green, then Yellow. In the description block Fantom3 refers to a non-coding database of Mus musculus that was submitted to the RNAdb (Pang et al. 2005). RNAz refers to non-coding RNA predicted to have function as assessed by Washietl et al. 2005. Evofold also refers to non-coding RNA predicted to have function as assessed by Pedersen et al. 2006.


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# Curriculum Vitae 

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