A New Model Army: Emerging fish models to study the genomics of vertebrate Evo-Devo

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Abstract

Many fields of biology – including vertebrate Evo-Devo research – are facing an explosion of genomic and transcriptomic sequence information and a multitude of fish species are now swimming in this 'genomic tsunami'.

Here, we first give an overview of recent developments in sequencing fish genomes and transcriptomes that identify properties of fish genomes requiring particular attention and propose strategies to overcome common challenges in fish genomics. We suggest that the generation of chromosome-level genome assemblies - for which we introduce the term 'chromonome' – should be a key component of genomic investigations in fish because they enable large-scale conserved synteny analyses that inform orthology detection, a process critical for connectivity of genomes. Orthology calls in vertebrates, especially in teleost fish, are complicated by divergent evolution of gene repertoires and functions following two rounds of genome duplication in the ancestor of vertebrates and a third round at the base of teleost fish.

Second, using examples of spotted gar, basal teleosts, zebrafish-related cyprinids, cavefish, livebearers, icefish, and lobefin fish, we illustrate how next generation sequencing technologies liberate emerging fish systems from genomic ignorance and transform them into a new model army to answer longstanding questions on the genomic and developmental basis of their biodiversity.

Finally, we discuss recent progress in the genetic toolbox for the major fish models for functional analysis, zebrafish and medaka, that can be transferred to many other fish species to study *in vivo* the functional effect of evolutionary genomic change as Evo-Devo research enters the postgenomic era.

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1. What fish can teach us about the evolution of vertebrate development

In both a numerical and a phylogenetic sense, asking about the genomic basis of fish Evo-Devo is the better part of asking about the genomic basis of vertebrate Evo-Devo. Not only are more than 50% of all living vertebrate species a 'fish' of some sort (Nelson, 2006), but also tetrapods are in fact specialized lobefin fish that happen to cope with life on land (Fig. 1). Casting hyperbole aside, dramatic recent progress in the genomic analysis of bony vertebrate (euteleostome) fish contributes substantially to our understanding of vertebrate Evo-Devo. This progress has been boosted by the revolution in 'next generation' sequencing techniques as well as major upgrades to the developmental genetic toolbox enabling the functional analysis of fish gene functions in an evolutionary framework.

Bony vertebrate fish have conquered almost every aquatic habitat on earth (and on earth in the form of tetrapods) and their biodiversity offers unmatched opportunities to study the genomic basis of adaptation, speciation, morphological and physiological change, and behavioral divergence. Among bony vertebrates, teleost fish are the most species-rich group (Nelson, 2006) and they offer unique advantages for the study of gene regulation, morphological evolution, and biodiversity at the functional level, taking advantage of methods developed for the developmental model teleosts zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*), (Furutani-Seiki and Wittbrodt, 2004) (Fig. 1).

The following discussion first illustrates recent progress in fish genomics and transcriptomics, highlighting specific challenges that come from applying nucleotide sequencing, assembly and annotation methods to fish genomes, then gives an overview of recent insights obtained from the genomic analysis of selected bony vertebrate fish models and the prospects these models offer for advancing understanding in the future. This overview emphasizes the diversity of fish genomes, which we are just beginning to understand. Finally, this review describes recent methodological advances and new strategies for analyzing the functional evolution of fish genes, mostly based on progress in molecular techniques for zebrafish and medaka, but advances that should be applicable to many other fish systems. Along the way, we will specifically stress the importance of genomic and functional resources that we consider integral to a comprehensive analysis of Evo-Devo genomics ('Evo-Devo-Geno') in emerging fish systems. These resources consist of genomic components (chromosome-level genome assemblies which we call 'chromonomes', high-resolution genetic maps, reference transcriptomes, and BAC libraries) in combination with the applicability of developmental approaches (access to spawns and developmental staging series, as well as methods for transgenesis and genome editing) (Fig. 1).

2. Sequencing and annotating fish genomes and transcriptomes

2.1 Assembly and annotation of fish gen(om)es: the apples-to-apples principle

Despite the large portion of vertebrate fauna that are rayfin fish, genome-sequencing efforts were until recently focused on a few phylogenetically isolated species. This cohort of five front swimmer species, sequenced mostly with classical Sanger methods, included the major developmental genetic model species zebrafish (Howe et al., 2013b) and medaka (Kasahara

et al., 2007), two species of pufferfish that were selected because of their small genome size (Aparicio et al., 2002, Jaillon et al., 2004), and threespine stickleback (Jones et al., 2012), the only species among the 'first wave' of fish genomes chosen predominantly to address evolutionary questions. As a result, one or a few of these genomic pioneers have been used as "the fish" in comparative studies with other vertebrate groups, an approach that falls short due to the diversity of rayfin – and especially teleost – genomes.

The recent revolution in sequencing methods has made possible the generation of genomic, transcriptomic, and epigenomic data at a speed and low cost undreamed of a decade ago. As rayfins are now swimming in the 'genomic tsunami', it has become almost impossible to keep track of fish genome projects. For recent censuses of ongoing fish genome projects see Bernardi et al. (2012) and Spaink et al. (2013). Many of the more recent fish genome initiatives are motivated by their interest for aquaculture and the fishing industry, such as Atlantic cod (Star et al., 2011), salmonids (rainbow trout: Berthelot et al., 2014; salmon: Davidson et al., 2010), and tongue sole (Chen et al., 2014), or as biomedical models for human disease (Albertson et al., 2009, Schartl, 2013) like platyfish (Schartl et al., 2013). Nevertheless, sequencing a rayfin fish genome just for the sake of tackling evolutionary developmental questions has become achievable for smaller research communities or even single labs, particularly considering the often relatively small size of fish genomes compared to tetrapod genomes.

Sequencing, assembling, and annotating rayfin genomes, and teleost genomes in particular, however, come with a specific set of pitfalls. Some factors such as the diversity of rayfin repetitive elements (Volff et al., 2003, Chalopin et al., 2013) or high rates of polymorphisms often found in fish genomes, even within inbred lab strains (Kasahara, et al., 2007, Brown et al., 2012), may specifically complicate the assembly of rayfin genomes (Bradnam et al., 2013). Another significant factor is that polyploidization, i.e., the duplication of the entire genome, is a common phenomenon, including famous examples such as salmonids, carps, and sturgeons (reviewed in Braasch and Postlethwait, 2012). Polyploidization may be a major obstacle for genome assembly, because the younger the genome duplication event, the more difficult it becomes to distinguish alleles from gene duplicates (paralogs).

A polyploidization event relevant to nearly all teleost genome analyses is the teleost genome duplication (TGD), which occurred in an ancestor of the teleost lineage (Amores et al., 1998, Taylor et al., 2003, Hoegg et al., 2004, Jaillon, et al., 2004, Crow et al., 2006, Hurley et al., 2007, Amores et al., 2011). Even though this polyploidization event is fairly old (~226–350 million years; Braasch and Postlethwait, 2012), it has an important influence on gene annotation, particularly on the determination of orthology, a prerequisite for any evolutionary meaningful analysis, i.e., comparing apples to apples or orthologs to orthologs. The evolutionary significance of the TGD for the diversification of the teleost lineage is a matter of ongoing debate (e.g., Donoghue and Purnell, 2005, Hurley, et al., 2007, Santini et al., 2009, Van de Peer et al., 2009), less controversial, however, is that it had a major impact on the evolution of teleost genome structure and teleost gene functions (Postlethwait et al., 2004, Braasch and Postlethwait, 2012). The rise of teleosts and the TGD have been associated with (i) a phase of major genomic rearrangement (Amores, et al., 2011), (ii) an increase in the molecular evolutionary rate, especially in non-coding regions (Ravi and

Venkatesh, 2008, Lee et al., 2011), and (iii) rediploidization of the tetraploid genome after the TGD, i.e., massive loss (nonfunctionalization) of genes and other genomic elements from a duplicated state back to a singleton state. In extant teleost genomes, around 20–25% of protein coding genes are still retained as two TGD paralogs (Braasch and Postlethwait, 2012), with important differences among functional gene classes with regard to the retention and loss of their TGD paralogs (Brunet et al., 2006, Kassahn et al., 2009, Howe, et al., 2013b, Schartl, et al., 2013). Genes of major concern for Evo-Devo research, such as transcription factor and other developmental genes are overrepresented among retained TGD paralogs (Brunet, et al., 2006, Kassahn, et al., 2009). Sequence divergence between TGD paralogs often occurs in an asymmetric manner, with one of the two TGD duplicates evolving faster than the other (Brunet, et al., 2006, Steinke et al., 2006). Furthermore, the processes of non-functionalization, rediploidization, and divergence in sequence and function often occur in a lineage-specific way (Postlethwait, et al., 2004, Semon and Wolfe, 2007, Braasch and Postlethwait, 2012).

Annotation of genomes and transcriptomes of 'non-model' fish species usually uses BLAST-based methods against the gene and protein sets of a 'model' species as a reference for which orthology assignments have already been generated (e.g., the 'front swimming five': zebrafish, medaka, stickleback, two pufferfish). This practice, however, is problematic because of lineage-specific reshuffling of teleost genomes following the TGD, which leads, for example, to the confusion of orthologs and paralogs (apples and oranges), the non-existence of orthologous genes due to reciprocal gene loss, the misassignment of GO terms, etc. This effect is exacerbated because zebrafish, the most detailed annotated teleost genome, as a cyprinid is phylogenetically isolated from most other sequenced teleost genomes (Hiller et al., 2013), including the many rising models belonging to the percomorphs (Fig. 1).

Problems with orthology assignment can often be solved on a gene-by-gene basis using a combination of phylogenetic methods and, if available, data on conserved synteny, i.e., conservation of gene arrangements. Generating phylogenies for every gene family present in entire genomes and transcriptomes [e.g. the EnsemblCompara Gene Trees (Vilella et al., 2009) generated during the Ensembl genome annotation process], remains challenging (Dessimoz et al., 2012). Often, phylogenetic data alone are insufficient to provide clear orthology assignments, especially in the case of fast and asymmetrically diverging teleost paralogs prone to all kinds of tree artifacts. In such cases, conserved synteny information becomes essential for inferring orthologies and the evolutionary origin of teleost genes (e.g., Postlethwait, 2007, Catchen et al., 2009, Catchen et al., 2011b). Although bioinformatic tools for the analysis of conserved syntenies are available [e.g., SyntenyDatabase (Catchen, et al., 2009); Gᴇɴᴏᴍɪᴄᴜs (Louis et al., 2013)], large-scale analyses of this kind require chromosome-level genome assemblies to perform. Most current second generation genome assemblies, however, remain fragmentary scaffold-level assemblies. To distinguish genome assemblies at the scaffold level from those at the chromosomal level, we introduce here the term 'chromonome' for the latter.

2.2 A call for fish 'chromonomes '

Chromonomes are essential for understanding genome evolution in the face of genome duplication with the associated problems of orthology assignment listed above. Fortunately, even for non-model organisms, the advent of second generation sequencing techniques enables the generation of high-density genetic linkage maps containing chromosome-length arrays of sequence-based markers along which the fragmentary scaffolds of a genome assembly can be aligned. An especially good method is restriction site associated DNA (RAD) marker sequencing, single nucleotide polymorphisms (SNPs) occurring in, say, 100 nucleotide long fragments sequenced adjacent to defined restriction sites (Miller et al., 2007, Baird et al., 2008). RAD sequencing (RAD-Seq) methods are now applied in variant forms on numerous types of genetic investigations, including population genomic and genome-wide association studies (GWAS), mapping of quantitative trait loci (QTL), mutant mapping, phylogenomics, and the generation of meiotic maps (see Davey et al., 2011, Hohenlohe et al., 2012 for recent overviews). RAD-Seq can provide thousands of polymorphic markers for the generation of genetic linkage maps (Amores, et al., 2011, Catchen et al., 2011a). The fact that many fish produce hundreds to thousands of embryos from a single cross makes RAD-Seq an ideal method to generate high-density linkage maps for any fish species for which a spawn can be obtained. RAD-seq is so efficient at identifying SNPs that one can usually abandon the former approach of mating widely divergent populations or even different species and making F2 or backcross populations, which was formerly the case, and now just take a single female and a single male from the wild, mate them, and directly obtain a map based on male and on female meiosis from F1 offspring. A meiotic map for the spotted gar was the first RAD-based linkage map of a rayfin (or of any vertebrate) (Amores, et al., 2011), and RAD-based linkage maps have since been generated for numerous other fish species including platyfish (Schartl, et al., 2013, Amores, et al., 2014), salmonids (e.g., Miller et al., 2012, Gagnaire et al., 2013), flatfishes (Palaiokostas et al., 2013a, Chen, et al., 2014), cichlids (Palaiokostas et al., 2013b, Recknagel et al., 2013), and Mexican tetra (O'Quin et al., 2013).

In the case of spotted gar, platyfish, tongue sole, and rainbow trout, RAD marker-based genetic maps were used to anchor draft genome assemblies onto linkage groups, generating chromosome-level genome assemblies, i.e., chromonomes. These chromonomes have enabled unprecedented insight into the evolution of teleost genome structure, sex chromosomes, and gene family histories (Amores et al., 2011, Schartl et al., 2013, Amores et al., 2014, Berthelot et al., 2014, Chen et al., 2014). Because teleosts are, on the one hand, particularly in need of large-scale conserved synteny analyses for orthology detection as result of the TGD and lineage-specific paralog losses, and are, on the other hand, well suited to generate high-density genetic maps due to their fecundity and ease of spawning, we propose that the generation of a RAD-based genetic map as part of a genome assembly project should become a standard in the field of teleost genomics. This 'call for chromonomes' will be a recurrent theme throughout the remainder of this article.

2.3 Fish transcriptomics and teleost GO terms

The evolution of RNA-Seq methods spurred a gold rush into sequencing transcriptomes and generating reference transcriptome assemblies for fish models (see Qian et al., 2014).

Generating *de novo* reference transcriptomes is a cost-effective way to obtain insight into the gene content of a non-model species, especially when the aim is to compare multiple species and when a reference genome assembly of a closely related species is unavailable. Nevertheless, with the dropping costs in genome sequencing and assembly, it may be worth considering putting efforts into generating a reference genome assembly first. Generally, reference-based transcriptome assembly strategies have several advantages and tend to be of higher quality compared to *de novo* assemblies generated without a reference genome (e.g., Martin and Wang, 2011, Vijay et al., 2013).

Numerous paralogs present in teleost genomes as a result of the TGD and the earlier vertebrate genome duplication events (see below) superimposed on lineage-specific gene losses cause particular problems in dealing with the annotation of teleost transcriptomes. The puzzling effect of lineage-specific paralog loss is particularly severe when a transcriptome is the only available source of gene sequence information, because missing content could be due either to insufficient transcript sampling or due to a true absence of a gene from the genome. Reciprocal failure to find paralogs in different lineages will give errors in orthology calling. As described above, taking conserved synteny information into account is often the only way by which orthology of teleost genes can be inferred, and conserved synteny data are by definition unavailable for transcriptomes. Thus, we suggest that a good strategy is to generate a reference genome assembly coupled to a meiotic linkage map of about 10,000 markers or more using about 100 or more map progeny, align the genomic sequencing scaffolds to the genetic map, and to construct a chromonome. This resource will inform the annotation of transcriptomic data, and reciprocally, a transcriptome will inform gene annotations for the genome.

Most large-scale gene expression analyses assign Gene Ontology (GO) terms to differentially expressed genes. GO terms for fish are unfortunately underdeveloped. Zebrafish is the best-curated species thus far, probably with a bias towards early developmental functions. Zebrafish, however, is phylogenetically distant from many teleost models (Fig. 1). Because of lineage-specific subfunctionalization after the TGD (Postlethwait, et al., 2004), one can assume that in some cases, specific subfunctions (potentially representing one or more GO terms) may have been retained in one TGD paralog in zebrafish, while being retained for the other TGD paralog in, for example, a percomorph. We currently don't understand how often reciprocal subfunctionalization occurs. A conservative strategy to improve putative GO term assignment in a fish species could thus be to use GO terms of both zebrafish paralogs, even if the orthology of the percomorph gene to one of the two zebrafish paralogs is unquestioned.

Besides the analysis protein-coding transcriptomes, efforts using RNA-Seq strategies are also underway in fish to identify and catalogue different types of non- coding RNAs, such as micro RNAs (miRNAs) and long non-coding RNAs (lncRNAs) (e.g., Pauli et al., 2012, Andreassen et al., 2013, Bizuayehu et al., 2013, Kitano et al., 2013). Zebrafish is here the best-documented fish species again, but non-coding RNAs from fish remain to be explored in detail and are currently undercurated in public databases like miRBase (Desvignes et al., 2014).

2.4 Don't give up on fish BAC libraries

A major leap forward for 'next-generation' genomes was the elimination of laborious bacterial cloning steps, making it seem that large insert size libraries such as fosmids and bacterial artificial chromosomes (BACs) had become obsolete. Sound reasons, however, suggest that investing in such types of libraries remains to be worthwhile. First, modified fosmids and BACs can be used as mate-pair libraries for Illumina-based genome assemblies (Williams et al., 2012), which assist with scaffolding efforts. Second, certain genomic regions, including highly repetitive sex chromosomes, are difficult to assemble from genomic DNA, and sequencing BAC clones tiling such regions may be an alternative strategy (Huddleston et al., 2014). Third, current genome assemblies are speckled with assembly gaps, which can be frustrating if such gaps are around your gene of interest, for example when investigating the presence/absence of a conserved non-coding element. Re-sequencing of the region from a BAC clone may help to close these gaps. And finally, BAC transgenesis in zebrafish and medaka has become an important method to study the *cis*-regulatory circuitry of a genomic region (see section 4). Therefore, we believe that BAC clones should remain an important component of the functional Evo-Devo-Geno toolbox.

3. Emerging fish systems for Evo-Devo-Geno research

In the following sections, we illustrate by the example of seven fish lineages how the new sequencing techniques provide insights into genome evolution of rayfin fish and the development of their diverse phenotypes. For each fish model, we will highlight recent progress as well as common problems that often apply to other fish systems. The narrative presents fish models according to their amenability to functional studies from routine (zebrafish) to impossible (coelacanth). In addition, Figure 1 and Table 1 give a brief overview of the genomic and developmental tools available for these species and other fish models and research questions for which various fish models are exquisitely poised. Detailed discussions of stickleback and cichlids, likely the most studied fish models in Evo-Devo-Geno research, are beyond the scope of the present article, but key articles on these two systems are listed in Table 1.

3.1 Basal rayfin fish and the benefits of the spotted gar model

What makes a teleost a teleost? What distinguishes teleosts from other rayfin fish? What is the secret to teleost evolutionary success? With more than 27,000 species, teleosts populate the majority of the rayfin fish fauna (Nelson, 2006), yet they are genomic outliers among bony vertebrates due to their paleopolyploid origin. To better understand the dynamics of genome evolution and gene function following the TGD, it is essential to define the ancestral, unduplicated state. Until recently, the only available well-studied outgroups diverging before the TGD were the tetrapods, especially mouse and human. The use of mouse as an ersatz unduplicated outgroup for the TGD was clearly just a makeshift solution because teleost and tetrapod lineages have experienced independent genomic and morphological evolution for the last 450 million years. A better outgroup to characterize the pre-TGD rayfin genome would be a 'model basal rayfin' species selected from the few extant non-teleost rayfin lineages (Fig. 2A): Polypteriformes (bichirs and reedfish), Acipenseriformes (sturgeons and paddlefish), and the lineage of Holostei consisting of

Amiiformes (bowfin) and Lepisosteiformes (gars). Major caveats exist, however, for the use of most of these lineages as genomic and/or functional rayfin basal models. Common to all, and a major challenge for use as laboratory model species, is their long generation times of usually several years.

Polypteriforms represent the most basal rayfin lineage and thus, on the one hand, are important models for the early events of rayfin evolution, but because of their very basal position they are, on the other hand, still relatively distant from teleosts and the TGD. In addition, polypteriform genomes tend to be larger than those of human (Takeuchi et al., 2009a). EST sequences for gray bichir (*Polypterus senegalus*) are available (Takechi et al., 2011), and analyses of bichir *hox* clusters, sequenced from BAC clones, suggest that they have a composition of *hox* genes and gene regulatory elements that is intermediate between teleosts and tetrapods (Chiu et al., 2004, Raincrow et al., 2011). Bichirs can be cultured and bred in the lab and are accessible to RNA *in situ* hybridization and microinjection (Takeuchi, et al., 2009a). Bichirs show many ancestral vertebrate features at the morphological and molecular level, including gene expression during early bichir development that has similarities to lamprey (Takeuchi et al., 2009b). Other morphological features of bichirs, however, such as fin structure, are derived in a polypteriform-specific manner not representative of the ancestral rayfin condition (Metscher and Ahlberg, 2001, Takeuchi, et al., 2009a), a disadvantage for understanding the origin of teleost-specific functions.

Sturgeons and paddlefish are common in aquaculture. Paddlefish is accessible for laboratory studies and has been used as outgroup in comparative studies to investigate, for example, *Hox* gene evolution during the fin-to-limb transition in lobefins (e.g., Davis et al., 2007, Crow et al., 2012). A problem for genomic analyses, however, is that Acipenseriformes have undergone several rounds of their own lineage-specific polyploidizations (Ludwig et al., 2001, Crow, et al., 2012). Therefore, the dynamics of their genome evolution, while interesting in themselves, make them represent a special case among rayfins and unsuitable as surrogates for the ancestral rayfin genomic condition. The morphological diversity of sturgeons has been underappreciated until recently (Rabosky et al., 2013), and it will be interesting to analyze if there are causal links between the multiple acipenseriform genome amplifications and morphological change in this group.

In the lineage most closely related to teleosts, the holosteans, the only living member of the amiiform lineage is the bowfin (*Amia calva*), which to the best of our knowledge has not been bred in captivity. Members of the family of Lepisosteiformes (gars) in the holostean lineage, in contrast, are of interest for aquaculture, and they can be consistently bred in captivity (Alfaro et al., 2008), making them available as laboratory animals for developmental studies. Work from our group has identified one of the smaller species, the spotted gar (*Lepisosteus oculatus*), as the most suitable model gar (Fig. 2B). Embryonic development of gars is relatively well described (Long and Ballard, 2001) and gars are suitable for RNA *in situ* hybridization, immunohistochemistry, and other developmental methods (e.g., Eames et al., 2012, Braasch et al., 2014). Importantly, spotted gar is well-suited to represent an approximation to the ancestral, pre-teleost and thus pre-TGD, developmental morphology. Ancestral morphological features of gars include their hardy

and protective ganoid scales, which share biochemical similarities with tetrapod tooth enamel (Sasagawa et al., 2013), a lung-like gas bladder used for air breathing (Longo et al., 2013), and a dorso-ventrally asymmetrical (heterocercal) tail rather than the pseudosymmetrical (homocercal), evolutionarily innovative tail of teleosts (Metscher and Ahlberg, 2001). Given its advantages, we proposed spotted gar as the best suited non-teleost rayfin fish model species (Amores, et al., 2011).

To investigate whether or not gars are indeed diploid and neither share the genome duplication common to all teleosts nor have their own lineage-specific genome duplication, a high-density genetic map for the spotted gar using RAD-Seq was generated (Amores, et al., 2011). By anchoring transcriptomic sequences derived from two map-cross embryos to the genetic map, it was possible to study patterns of conserved synteny between gar and tetrapods as well as gar and teleosts, even without a reference genome (Amores, et al., 2011). This work has thus provided definitive proof that the TGD happened after the divergence of teleosts from holosteans and is therefore a teleost-specific event. Furthermore, the mapping work showed that genome rearrangements occurred more frequently in teleosts compared to tetrapods and gar (Amores, et al., 2011; Fig. 2C). The genome of the spotted gar has since been sequenced and anchored onto the genetic map and is available in Ensembl [http://www.ensembl.org/Lepisosteus_oculatus].

The spotted gar chromonome is an important resource not only for the study of genome evolution in teleosts, but also for the investigation of the ancestry of bony vertebrate gene functions in general. Following the 'genomic big bang' of the earlier rounds of whole genome duplication at the base of the vertebrate lineage, VGD1 and VGD2 (Fig. 1) (reviewed in Canestro, 2012), and subsequent lineage-specific genome reorganization and rediploidizations, the genomes of agnathan, cartilaginous, and bony vertebrates and the gene repertoires they encode have significantly diverged. Gene duplicates that originate in genome duplications are called "ohnologs" (Wolfe, 2001). Following VGD1 and VGD2, many genes were present in four ohnologous copies organized in four ohnologous regions (ohnologons, like the four Hox clusters and many surrounding genes) that are still apparent in extant vertebrate genomes (up to eight in teleosts following the TGD) (reviewed in Canestro, 2012). Gene families with four remaining VGD1/2 ohnologs are, however, rather rare and VGD1/2 ohnologs have frequently gone missing in a lineage-specific manner. Therefore, it often becomes difficult to interpret orthology of genes across vertebrate lineages and to detect instances of 'hidden paralogy' among vertebrate genes (Kuraku, 2013). Lineage-specific 'ohnologs gone missing' may not only confound interpretations of functional gene evolution across vertebrate lineages (Braasch and Postlethwait, 2012), but can also lead to situations in which orthologous genes simply no longer exist to study the ancestry of gene functions. For example, numerous genes have been retained in teleosts and the lobefin coelacanth but were lost during the water-to-land transition in tetrapods (Amemiya et al., 2013). Likewise, some VGD ohnologs may have been lost in teleosts but retained in tetrapods (e.g., *Pou5a1/Oct4*, Frankenberg and Renfree, 2013; *Prrx2*, Braasch, et al., 2014) so that studying their ancestral function in fish cannot be modeled with zebrafish or medaka, requiring a model that has retained many ancestral ohnologs and is amenable to

functional studies. While coelacanth is not available for functional studies and lungfishes are only rarely available (see section 3.7), spotted gar can fill this gap (Braasch, et al., 2014).

3.2 Genomic diversity of basally diverging teleost lineages

Shortly after the TGD event, the teleost lineage split into three major branches (Figs. 1, 2A): clupeocephalans (encompassing the majority of teleost diversity, including the radiations of the Ostariophysi and Percomorpha), elopomorpha (eels, tarpons, tenpounders, bonefish), and osteoglossomorpha (bony tongues, goldeye, etc.). Unfortunately, available teleost genome assemblies are almost exclusively from clupeocephalan teleosts. For elopomorphs, draft genome assemblies for two species of eel, European eel (*Anguilla anguilla*) and Japanese eel (*A. japonica*), are available (Henkel et al., 2012a, Henkel et al., 2012b). Transcriptome data from the European eel (Coppe et al., 2010, Ager-Wick et al., 2013) and a genetic map for the Japanese eel based on microsatellites and AFLP data (Nomura et al., 2011) are available as well. Developmental studies of eels are hindered by their enigmatic, catadromous life styles that start with spawning in the open ocean, impeding artificial breeding efforts – a major drawback considering the economic importance of eels (Henkel, et al., 2012a, Henkel, et al., 2012b).

Osteoglossomorphs remain a large blind spot for teleost genomics. The superorder Osteoglossomorpha consists of two orders, Osteoglossiformes (bonytongues) and Hiodontiformes (mooneyes) (Nelson, 2006). No genome assembly is currently available for any osteoglossomorph. A low-density genetic map and a first transcriptome have been recently published for the Asian arowana (*Scleropages formosus*) (Shen et al., 2013a). For Hiodontiformes, a few genes including *hox* cluster genes of the goldeye (*Hiodon alosoides*) have been analyzed through PCR screens (Hurley, et al., 2007, Chambers et al., 2009).

Despite the current paucity of elopomorph and osteoglossomorph genomic data, we can deduce a few early trends for the basally diverging teleosts:

i. Clupeocephalans, elopomorphs, and osteoglossomorphs differ markedly in their retention of ancestral VGD1/2 ohnologs. While many *hox* paralogy groups are missing from clupeocephalans, eels have kept almost all of them intact (Henkel, et al., 2012a, Henkel, et al., 2012b). Another example, the *prrx2* gene (a VGD1 ohnolog of *prrx1*) is absent from clupeocephalans, but present in elopomorphs (eels) and osteoglossomorphs (butterflyfish), showing that *prrx2* is not a pan-teleost ohnolog gone missing (Braasch, et al., 2014) as was initially assumed.

ii. The three major teleost lineages differ in their retention of TGD paralogs. Even clupeocephalans show significant diversity between zebrafish and percomorphs (medaka, stickleback, pufferfishes, etc.) in terms of TGD paralog retention and loss, although the overall level of TGD paralog retention is similar among extant clupeocephalan genomes (e.g., see Braasch et al., 2009, Sato et al., 2009, Schartl, et al., 2013). It can be expected that the diversity in TGD paralogs is even more pronounced when comparing the three early diverging teleost lineages. Indeed, while most clupeocephalan lineages have reduced their *hox* complement to seven hox clusters, eight *hox* clusters are present in eels (Henkel, et al., 2012a, Henkel, et

al., 2012b) and the osteoglossomorph goldeye (Chambers, et al., 2009). *Vice versa*, some genes appear to be single copy in elopomorphs and/or osteoglossormorphs, while having been retained in two copies after the TGD in clupeocephalans, e.g., *sox9* (Shen, et al., 2013a) or *pomc* (see below).

iii. According to nomenclature conventions, '*a*' and '*b*' suffixes are added to teleost gene names for TGD paralogs. While calling orthologs of the *a*- vs. *b*-paralogs is often straightforward among clupeocephalans (e.g., comparing zebrafish to medaka), phylogenetic methods frequently fail in assigning the *a/b*-orthology of clupeocephalans to elopomorphs and osteoglossomorphs. An example for this phenomenon, the *pro-opiomelanocortin* (*pomc*) gene, is shown in Fig. 3. Clupeocephalans have two *pomc* TGD paralogs, called *pomca* and *pomcb* (de Souza et al., 2005). In contrast, eels have a single *pomc* gene (Ager-Wick, et al., 2013). From pilot genome sequencing, we identified only one *pomc* gene for goldeye (I. Braasch, P. Batzel, A. Amores, J. H. Postlethwait, unpublished data). This result suggests that eels (elopomorphs) and maybe goldeye (osteoglossomorphs) lost one of the two TGD *pomc* paralogs at some point during their evolution following the TGD. Phylogenetic reconstructions, however, fail to assign orthology of the single remaining *pomc* gene of these two basal teleost lineages to one of the two *pomc* TGD paralogs of clupeocephalans. Likewise, we cannot tell whether the eel *pomc* gene is the ortholog or a TGD paralog of goldeye *pomc* (Fig. 3A). Even analysis of small-scale conserved syntenies doesn't help in this case because, although the direct genomic environment of eel and goldeye *pomc* genes has been sequenced, these scaffolds are too short to infer whether these gene regions are more closely related to the cluepocephalan *a*- or *b*-paralogons by means of conserved syntenies (Fig. 3B). Other examples for unclear orthologies of elopomorph/osteoglossormoph and clupeocephalan genes include many *hox* TGD paralogs in eel and goldeye (Chambers, et al., 2009, Crow et al., 2009, Henkel, et al., 2012a), *sox9* in the osteoglossomorph arowana (Shen, et al., 2013a), and *prrx1* paralogs in elopomorph eels and the osteoglossomorph butterflyfish (Braasch, et al., 2014). Additional measures such as splicing patterns and/or long-range conserved syntenies need to be taken into account for orthology detection of elopomorph/osteoglossomorph *vs.* clueocephalan genes (Braasch, et al., 2014). Even these measures may not necessarily provide certainty because TGD paralogs and paralogons of clupeocephalans and the other two lineages could also evolve convergently following the TGD.

These early trends have important implications for teleost genomics. The example of *pomc* genes illustrates the need for long-range, chromosome length synteny information for elopomorphs and osteoglossomorphs in the form of chromonomes. Only with chromonomes will we be able to fully appreciate the diversity of teleost genomes and infer the early events of teleost genome evolution shortly after the TGD. Chromonomes will enable tests of the 'genomic big bang hypothesis' that the three teleost 'genome galaxies' (elopomorphs, osteoglossomorphs, and clupeocephalans) have been drifting apart in genome evolutionary space since the TGD, potentially leaving few genomic commonalities behind. Such analysis is important because it will illuminate general principles that also characterized the

aftermath of the more ancient VGD1/2 genome duplications at the base of the vertebrate radiation that shape the genomes of all vertebrates (including our own), which due to their even deeper ancestry and paucity of surviving, anciently diverging lineages are even more difficult to study.

The unfolding diversity of teleost genomes has additional relevance for phylogenomic analyses of basal teleost relationships. Based on morphological and molecular data, three hypothesis have been discussed (Li et al., 2008): (1) Osteoglossomorphs are the sister lineage of all other teleosts: (osteoglosso (elopo+ clupeo)); (2) Elopomorphs are the sister lineage of all other teleosts: (elopo (osteoglosso + clupeo)); (3) Clupeocephalans are the sister lineage of a clade combining osteoglosso- and elopomorphs: (clupeo (osteoglosso + elopo)). While the basal position of osteoglossormorphs (hypothesis 1) is supported by phylogenies of mitochondrial genomes (Inoue et al., 2003, Setiamarga et al., 2009), several recent multi-locus phylogenomic studies based on 10–20 nuclear marker genes support the basal divergence of elopomorphs (hypothesis 2) (Near et al., 2012b, Betancur et al., 2013, Broughton et al., 2013). Nuclear markers used in these studies were designed from comparative genomic screens for singleton genes of zebrafish and pufferfish and other percomorphs (Li et al., 2007). In using these markers, the studies assume that the markers are singletons in all teleosts, that the loss of their TGD paralogs occurred shortly after the TGD and predate the divergence of living teleost lineages, and that these markers are thus strictly orthologous. Reciprocal loss of TGD paralogs, i.e., loss of the *a*-paralog in one lineage and loss of the *b*-paralog in another lineage leaving paralogous and not orthologous singletons behind, appears to be rather rare within clupeocephalans (<10% between zebrafish and percomorphs; Semon and Wolfe, 2007, Kassahn, et al., 2009). The unfolding diversity of basally diverging teleost lineages described above, however, suggest that singletons in one lineage (clupeocephalans in this case) may not necessarily be singletons in the other two major lineages (elopomorphs, osteoglossormophs). Furthermore, even if a marker gene is present in only one copy in all three teleost lineages, these singletons may not necessarily be true orthologs and could be – at an unknown frequency – instances of 'hidden paralogy' (Kuraku, 2013). Hidden paralogy becomes more likely the shorter the delay between the TGD event and the divergence of the three teleost lineages, and current studies suggest that these incidents happened within a short time window of a few tens of millions of years (Near, et al., 2012b, Broughton, et al., 2013). Future studies therefore will need to address whether or not the assumptions made in the use of singleton nuclear markers developed for clupeocephalans are met in the other two teleost lineages and assess how potential violations of these assumptions may have influenced recent conclusions about the relationships of basally diverging teleost lineages. Such assessment, again, will require large-scale conserved synteny data and thus chromonomes from elopomorphs and osteoglossomorphs to improve orthology calls among the three teleost lineages.

3.3 The *Danio* flotilla and the cyprinid armada

Cyprinidae is the most diverse family in all of Vertebrata with more than 2,400 species and 200 genera. This group includes some of the best-known fish in the world such as carps, goldfish, and zebrafish, the most widely used teleost developmental model organism. The

wealth of cyprinid biodiversity has spawned projects such as the Cypriniformes Tree of Life [http://bio.slu.edu/mayden/cypriniformes/overview.html].

A number of cyprinids, including many of the seventeen currently recognized *Danio* species, display characteristics that make the group of zebrafish and related species, the danionins, interesting as an Evo-Devo model species complex. Phenotypic differences among danionins include both discrete changes (e.g. loss or fusion of craniofacial elements, reduction and loss of barbels and breeding tubercles), and more complex changes (e.g., reduction of adult size, mineralization, and diverse pigmentation patterns) (Fang, 2003, Parichy, 2007). Danionins and other related cyprinids further allow the investigation of repeated evolution of miniaturization in this group (Ruber et al., 2007). Two zebrafish-related species of note are *Paedocypris progenetica*, which holds the record as the smallest known fish (Kottelat et al., 2006), and *Danionella dracula*, which grows evolutionarily novel "fangs" on its jaws as its name suggests (Britz et al., 2009). The most obvious and well-studied differences between *Danio* species and related genera, however, are found in their diversity of adult coloration and striking pigment patterns, some of which resemble zebrafish pigmentation mutants (Parichy, 2007). Clever complementation tests in interspecies hybrids derived from these zebrafish mutants and other *Danio* species have helped to elucidate the signaling pathways involved in the differentiation and evolution of pigment patterns across the genus (Parichy and Johnson, 2001, Quigley et al., 2005). Follow up studies in zebrafish have subsequently explored cellular interactions of different types of pigment cells leading to stripe formation (Inaba et al., 2012, Hamada et al., 2014) and the genetic mechanisms causing pigment cell differentiation (Patterson and Parichy, 2013).

These studies illustrate three major strengths of the danionins for studies in evolution and development. First, these species are able to hybridize, bringing together genomes separated by millions of years in a nested series of evolutionary time scales and allowing access to the wealth of naturally occurring genetic diversity that has arisen as the species diversified. Interspecies hybrids will allow one to investigate the genetic and developmental basis of this variation as the genomes of two different species interact sharing the same cell nucleus within single individuals. Second, the unmatched infrastructure for genetic analysis and imaging of zebrafish development as well as ease of laboratory culture allows for investigation of the genomic and cellular mechanisms underlying the evolution of development of the danionins. Third, the zebrafish reference chromonome (Howe, et al., 2013b), along with accessibility of zebrafish mutants for a multitude of genes (Kettleborough et al., 2013), transgenic lines, and the curation of zebrafish research data by ZFIN, the Zebrafish Information Network (Howe et al., 2013a) allows for follow-up experiments to functionally disentangle the genetic and cellular basis of evolutionary change in the *Danio* species complex (see also section 4). The danionins are therefore ripe to become the 'drosophilids' of vertebrate Evo-Devo-Geno research.

Beyond the *Danio* system, the species-richness of the cyprinid family provides a wealth of natural genetic and phenotypic variation for study in an Evo-Devo framework. To this end, the PHENOSCAPE project aims to extend the genetic and developmental knowledge gained from zebrafish to cyprinids and Ostariophysi (Fig.1) to develop explicit Evo-Devo hypotheses (Mabee et al., 2012).

Cyprinidae also embraces the subfamily Cyprininae, which includes common carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*), the oldest domestication experiments of the fish world. In addition to the TGD, this group underwent an additional whole genome duplication event within the last few million years (Ohno et al., 1967, Wang et al., 2012). This recent carp genome duplication allows for the study of post-duplication genome evolution on a different timescale than the basal teleost models mentioned previously. Striking phenotypes in goldfish and carps are the result of centuries of artificial selection and represent another potential avenue of interest for Evo-Devo research, similar to domestication in dogs or pigeons. Historical relationships among various goldfish strains have only recently been determined and the genomic basis of color patterns and morphological monstrosities like bubble eyes, pearlscale, or lionhead remains to be uncovered (Komiyama et al., 2009, Wang et al., 2013).

The importance of carps in aquaculture has resulted in numerous studies of gene expression and physiology, genetic maps, and a recent first genome assembly for common carp (Henkel et al., 2012c). Carp is the second available cyprinid genome draft after zebrafish, now allowing, for example, the inference that the high repeat content in zebrafish is not common to cyprinids in general, because the genome of carp – despite of its tetraploid origin – is similar in size to zebrafish thanks to a much lower repeat content (Henkel, et al., 2012c). Syntenies appear to be almost perfectly conserved between carp and zebrafish (Henkel, et al., 2012c), as well as between the zebrafish and a RAD-based genetic map of gudgeon (*Gnathopogon*), another cyprinid (Kakioka et al., 2013). A second cyprinid chromonome, however, will be necessary to evaluate in detail whether the increase in interchromosomal rearrangements found for zebrafish compared to percomorphs (Braasch and Postlethwait, 2012, Howe, et al., 2013b) is a pervasive feature of cyprinids in general or a specific phenomenon of the zebrafish lineage.

3.4 Cavefishes: convergent evolution in the dark

Cavefish populations of the Mexican tetra (*Astyanax mexicanus*) are paradigmatic examples for evolutionary change in an extreme environment: constant darkness. *Astyanax* cavefish (also known as 'blind cavefish') are characterized by a combination of 'regressive' and 'constructive' changes including the reduction of eyes and pigmentation (albinism), changes to their sensory organs, central nervous system, and craniofacial structures, modified feeding behavior, loss of sleep and schooling behaviors and many more. The blind cavefish is thus an ideal fish system to study the genomic basis of vertebrate Evo-Devo at the microevolutionary scale (reviewed in Jeffery, 2008).

Fertile hybrids between cave and ancestral surface populations allow extensive QTL mapping analyses to identify genetic loci that differentiate cave from surface forms. These studies revealed a complex genomic architecture of cavefish evolution, with multiple loci underlying regressive and constructive traits (e.g., Protas et al., 2006, Protas et al., 2007, Yoshizawa et al., 2012, Kowalko et al., 2013, Gross et al., 2014). Population genomic analysis based on RAD-seq data further suggests that standing genetic variation significantly contributed to the evolutionary changes observed in cave populations (Bradic et al., 2013). This hypothesis gets further support by the observation that experimental inhibition of stress

response as well as water conditions found in caves can unmask cryptic eye variation in surface fish (Rohner et al., 2013).

The question of whether regressive phenotypes in cavefish, like loss of eyes and pigmentation, are based on neutral processes and drift or whether they are adaptive and under positive selection, possibly as an indirect effect of selection for pleiotropic genes, is a hotly debated matter (see e.g., Jeffery, 2009, Wilkens, 2010, Retaux and Casane, 2013). For example, it has been suggested that cavefish eye loss could be an indirect effect of selection for increased sensory neuron numbers enabling adaptive feeding behavior in the dark using vibration response (Yoshizawa, et al., 2012). Inhibition of pigment synthesis on the other hand has been proposed to increase tyrosine levels in albinistic cavefish. Because tyrosine is a precursor of both melanin pigments and neurotransmitters, cavefish albinism may thus be adaptive by making additional tyrosine available for neurotransmitter synthesis, thereby contributing to complex cavefish behaviors (Bilandzija et al., 2013).

Astyanax cavefish is well suited for functional studies due to the availability of embryos from different populations and its relatively close phylogenetic relationship with zebrafish (Fig. 1), which has helped in the cloning of candidate genes for functional tests and adopting much of the zebrafish methodological toolkit to *Astyanax* research (Jeffery, 2008). As described in section 4, functional studies on gene candidates underlying cavefish QTLs have led to the identification of the genomic basis of cavefish pigmentation phenotypes. While albinism is caused by mutations in *oca2* (Protas, et al., 2006), mutations in the *mc1r* gene are responsible for the 'brown' phenotype (Gross et al., 2009). Candidate gene expression studies furthermore identified the hedgehog signaling pathway as a key player for eye regression in cavefish. Expanded expression of *sonic hedgehog* along the anterior midline was observed in cavefish compared to surface fish, inducing cavefish eye regression through lens apoptosis, but also leading to advantageous increases in jaw size and taste bud numbers (Yamamoto et al., 2004, Yamamoto et al., 2009) – further indication for the potential linkage of regressive and constructive traits. Surprisingly, *shh* or other components of the hedgehog pathway do not locate to the QTL regions involved in eye regression suggesting that these loci may contain yet unidentified upstream regulators of hedgehog signaling (Protas, et al., 2007, Jeffery, 2009, Yoshizawa, et al., 2012).

Transcriptomic analyses of *Astyanax* cave *vs*. surface fish further highlight cavefish-specific mutations and expression differences in the visual system and metabolic pathways (Gross et al., 2013, Hinaux et al., 2013). The *Astyanax* genome assembly (not yet available as a chromonome) became available recently [http://www.ensembl.org/Astyanax_mexicanus] and represents an important step for teleost genomics in general because it is the first non-cyprinid genome available from the superorder Ostariophysi, which accounts for almost one-third of known fish species (Fig. 1) (Nelson, 2006). The impressive recent advances in detailed genetic analyses, genomic resource availability, and its functional accessibility establish the *Astyanax* system as a fully equipped 'model system' to study vertebrate Evo-Devo.

In addition to *Astyanax* about 150 other fish species live in cave environments (Retaux and Casane, 2013), offering avenues for asking about the convergent evolution of cave-dwelling

fish traits. Current evidence points towards different molecular evolutionary trajectories that can lead to convergence in cave phenotypes in fish. For example, important differences in the molecular evolution of the circardian system have been found between *Astyanax* cavefish and the Somalian cavefish (*Phreatichthys andruzzii*), a cyprinid (Beale et al., 2013). Furthermore, many cave species are found among goldenline barbels, cyprinids of the genus *Sinocyclocheilus* from China, that are further characterized by conspicuous horns and humps of mostly unknown function (Zhao et al., 2011) (Fig. 1). Goldenline barbels display important differences in the cellular mechanism for eye regression compared to *Astyanax*. While *shh*-induced apopotosis of the lens causes eye regression in *Astyanax* (Yamamoto, et al., 2004, Jeffery, 2009), such lens degeneration is not observed in the *Sinocyclocheilus* cavefish, which instead show regressive changes to the retina of their small, internalized eyes (Meng et al., 2013a). Transcriptomic comparisons of cave and surface eyes in *Sinocyclocheilus* further support the hypothesis of retinal degeneration as photoreceptor-specific genes such as opsins and their common upstream regulator Crx are downregulated in *Sinocyclocheilus* cavefish (Meng, et al., 2013a, Meng et al., 2013b). The molecular genetic mechanism leading to lower *crx* expression in cavefish eyes remains to be elucidated. Comparing gene expression of cave *vs.* surface forms of *Sinocyclocheilus* and *Astyanax* revealed few expressional changes shared between the two lineages. This finding suggests that different molecular mechanisms lead to convergent evolution of eye regression in *Sinocyclocheilus* and *Astyanax* (Meng, et al., 2013a).

3.5 Life in Technicolor: poeciliid livebearers

The family of Poeciliidae, also known as 'livebearers', has been studied for more than a century for their amazing morphological adaptations and phenotypic variation (see Evans et al., 2011 for an overview). The family contains some of the most common aquarium species: guppy, mollies, platyfish, and swordtails. The first sequenced poeciliid genome is that of the platyfish (*Xiphophorus maculatus*), a chromonome build by the inclusion of a RAD-based high-density genetic map (Schartl, et al., 2013, Amores et al., 2014). The platyfish chromonome highlights the stability of percomorph karyotypes because only few interchromosomal rearrangements were found between platyfish, medaka, stickleback, and pufferfish (Schartl, et al., 2013). Transcriptomes of other poeciliid species, such as additional species of the genus *Xiphophorus* (Cui et al., 2013, Schartl, et al., 2013, Shen et al., 2013b) or the guppy (*Poecilia reticulata*) (Fraser et al., 2011), have become available as well and multiple poeciliid genome assemblies are in progress.

As implied by their common name, all but one of the more than 260 species of livebearers are characterized by their viviparous reproduction mode; species across the group show a spectrum of continuous complexity in maternal provisioning, including the evolution of placentas (Pollux et al., 2009). A first avenue towards understanding the evolution of viviparity in poeciliids comes from sequence comparison of viviparity candidate genes from the platyfish genome and *Xiphophorus* transcriptomes with those of other teleost species leading to the identification of several viviparity candidate genes under positive selection in poeciliids (Schartl, et al., 2013).

Poeciliid sexual dimorphism is seen in many morphological structures, most prominently in the male gonopodium, a transformed anal fin used as intromittent organ for internal fertilization, as well as in the ventral sword-like caudal fin extension in swordtails of the genus *Xiphophorus*. Considering swordtails as a model for the evolution by sexual selection actually dates back to Darwin's *Descent of Man, and Selection in Relation to Sex* (1871) (Darwin, 1871). Recent large-scale phylogenomic analysis based on RAD markers (Jones et al., 2013) and transcriptomes from multiple *Xiphophorus* species (Cui, et al., 2013) support the hypothesis that the development of a sword in *Xiphophorus* males is the ancestral state in the genus and that the sword has been lost secondarily most likely more than once in the genus.

Another striking phenotype of poeciliids – and of platyfish and guppy in particular – that has fascinated scientists for a century is their diversity in color patterns, which are often sexually dimorphic and to a large degree based on sex- linked loci (Winge, 1927, Basolo, 2006). While the genetic basis of these sex-linked color loci remains largely unresolved, the platyfish chromonome excluded the vast majority of classic pigmentation genes as candidates for these loci because they were found not to locate to the platyfish sex chromosomes (Schartl, et al., 2013). In contrast, the receptor tyrosines kinase genes *kit and csf1r*, well known from zebrafish pigment mutants and their involvement in pigment pattern divergence among *Danio* species (Parichy, 2007), were identified as the genomic bases of two autosomal color loci in guppy (Kottler et al., 2013).

Sex chromosomes are generally genomic hotspots for accumulating loci contributing to poeciliid diversity. While poeciliid sex determination genes remain to be identified, the genetic basis of some sex-linked loci has been elucidated. The classic example is a tumor locus that causes the development of melanoma in interspecific *Xiphophorus* hybrids. These skin cancers are caused by *Xmrk*, an oncogenic, sex-linked, local gene duplicate of the receptor tyrosinase gene *egfrb* (Meierjohann and Schartl, 2006); *Xmrk* has been proposed to represent a *Xiphophorus* 'speciation gene' (see Schartl, 2008 for a critical discussion). The sex-linked 'puberty locus' determining the onset of male maturity and mating behavior in *Xiphophorus* appears to be caused by copy number variation of multiple *melanocortin 4 receptor* (*mc4r*) gene duplicates that were identified by sequencing BAC clones from the highly repetitive platyfish sex chromosomes (Lampert et al., 2010, Volff et al., 2013).

Poeciliids are popular models to study teleost behaviors. Coupled with complex behavior, genes implicated in cognition are retained at a particularly high rate, around 45% compared to only around 15% for liver genes, as became apparent from comparisons of the platyfish gene content to that of other teleosts and mammals (Schartl, et al., 2013). Likewise, the zebrafish genome project showed a high TGD duplicate retention for neuronal genes (Howe, et al., 2013b). These findings suggest that highly complex cognitive abilities and behaviors found in teleosts may be based on functional divergence of gene duplicates retained from the TGD.

Functional studies in poeciliids are hampered by their livebearing lifestyle that inhibits genetic manipulations and transgenesis. The recent progress in poeciliid genomics, in combination with functional approaches such as testing poeciliid gene functions in medaka

(section 4) thereby serves as a paradigm for other fish systems that are challenged by internal development such as sygnathids (pipefishes, sea horse, sea dragons) or mouthbrooding cichlids.

3.6 Adaptation to the cold: Antarctic rockcods and icefish

Antarctic fish constitute an Evo-Devo model of many extremes because of their polar habitats and the technical challenges that accompany this extreme and remote environment. In the freezing cold waters of the Southern Ocean (−1.9°C), Antarctic fish diversity is largely dominated by a single suborder, the notothenioids, which form a remarkable example of adaptive radiation in an extreme cold environment (Eastman, 2005). As Antarctic waters cooled, which reaching their current frigid conditions about 10–14 Myr ago (Kennett, 1977), many taxa became locally extinct, liberating various ecological niches (Eastman, 1993, Eastman and McCune, 2000) that notothenioids were able to conquer based on dramatic adaptive changes towards cold tolerance and the use of new habitats.

The most striking example of adaptive evolution to frigid environment is the gain of antifreeze glycoproteins (AFGPs) that evolved from pancreatic trypsinogen and allowed notothenioids to survive, adapt and diversify in freezing waters in which other species would freeze to death (Cheng and Chen, 1999, Kock, 2005b, Kock, 2005a, Cheng and Detrich, 2007). Several other molecular and cellular changes accompanied the adaptation to a constant icy-cold environment including an apparent loss of inducible heat shock response in steady cold ambient temperature (Hofmann et al., 2000, Buckley et al., 2004, Place and Hofmann, 2005, Huth and Place, 2013).

With the development of transcriptomic techniques, recent studies have looked at the effects of acclimation temperature and heat-shocks on gene expression in several species of cold-adapted notothenioids and suggested (i) numerous gene duplications (Chen et al., 2008, Coppe et al., 2013) consistent with large genome size (Detrich et al., 2010), (ii) the involvement of the ubiquitin-conjugated protein cytosolic protein-degradation pathway of misfolded or damaged protein as putative factor of cold adaptation (Shin et al., 2012, Bilyk and Cheng, 2013, Huth and Place, 2013), and (iii) increased mitochondrial function (Coppe, et al., 2013).

Being able to survive in icy waters, notothenioids diversified from a benthic scavenger common ancestor to species inhabiting all parts of the water column spanning from benthic to pelagic habitats (Eastman, 1993). Although all notothenioids lack a swim bladder, the diversification along the vertical water column and evolution to become ambush feeders was accompanied by buoyancy modifications (Kock, 2005a) (Fig. 4). Among changes, the most striking ones are the delay and reduction of skeletal ossification through the alteration of collagen gene expression (Albertson, et al., 2009), the loss or reduction of mineralized elements such as scales and dermal bones (Kock, 2005a, Albertson, et al., 2009), and the evolution of extensive unique lipid deposits (Friedrich and Hagen, 1994, Hagen et al., 2000). These evolutionary alterations reflect paedomorphic changes by the retention of larval characteristics in the adult.

One synapomorphy of the family of icefishes, the Channichthyidae (Kock, 2005b, Kock, 2005a), has baffled biologists from the first report by J. T. Ruud in 1954 of "Vertebrates without erythrocytes and blood pigment" (Ruud, 1954). This puzzling condition, lethal in any other vertebrate species, was shown to be a disadvantageous but non-lethal ('disaptive') phenotype (Baum and Larson, 1991, Montgomery and Clements, 2000) at low temperature because oxygen concentration is inversely correlated with water temperature. Furthermore, globin-independent loss of cardiac myoglobin has occurred in six of sixteen species of Channichthyidae (Sidell et al., 1997, Cheng and Detrich, 2007) (Fig. 4). The loss of ability to express globin and myoglobin genes was compensated by molecular and morphological adaptations such as diffusion of oxygen through the scale-less skin of icefishes and modest decrease in metabolic oxygen demand (Hemmingsen, 1991), an increased heart size and pumping volume in relation to body size (Hemmingsen, 1991), a more developed vascular system (Egginton et al., 2002), and quantitative changes in mitochondrial density and morphology (Sidell, et al., 1997, O'Brien et al., 2000).

Interestingly, these oxygen-carrier-lacking vertebrates could help understand molecular mechanisms of erythropoiesis and anemia (Albertson, et al., 2009). BAC libraries from erythrocytes of the red-blooded Yellow belly rock-cod *Notothenia coriiceps* and from leukocytes of the clear-blooded Blackfin Icefish *Chaenocephalus aceratus* have been generated (Detrich and Amemiya, 2010, Detrich, et al., 2010). Representational Difference Analysis of cDNA libraries from pronephric kidney of the same two species identified a novel gene, *bloodthirsty*, that was further shown to play a role in differentiation of the committed red cell progenitor by functional analysis in zebrafish (Yergeau et al., 2005).

The Antarctic notothenioids represent a compelling opportunity to study morphological, physiological and molecular evolutionary development adaptations of a marine species-flock to freezing cold environment. Breeding and raising notothenoids, however, is technically difficult and is currently only possible at Antarctic research stations. Therefore, a combination of genomic and transcriptomic characterization of notothenoids in combination with functional tests within other fish species - as exemplified by the *bloodthirsty* case (Yergeau et al., 2005; see also section 4) - appears to be the method of choice to unravel the extraordinary morphological and physiological adaptations of these element-braving fish.

3.7 Coelacanth and lungfish: how lobefin fish left the water

Probably the most iconic fish genome sequenced so far is that of legendary 'Old Fourlegs', the coelacanth (*Latimeria*) (Amemiya, et al., 2013, Nikaido et al., 2013). Coelacanths were thought to be extinct until their rediscovery in 1938 and since then have been considered 'living fossils' (Smith, 1939). Coelacanths and lungfishes (Dipnoi) represent one of the only two living lobefin (sarcopterygian) lineages that are fish but not tetrapods (Fig. 1). Therefore, coelacanths and lungfishes are essential to understand the conquest of land and the morphological changes that occurred during the water-to-land transition, especially the fin-to-limb transition. The question of whether coelacanths or lungfishes represent the sister lineage tetrapods has been long debated. Lungfish have the largest genomes of the animal kingdom – estimates range from 40 to 130 Gb – presumably due to massive amplification of repetitive elements early in the lungfish lineage that are now 'fossilized', escaping current

repeat detection methods (Metcalfe et al., 2012). These gigantic genomes make it impractical to sequence a lungfish genome and transcriptomic characterization of the lungfish gene content is currently the only practical option. Phylogenomic analysis of the coelacanth genome content in combination with gene sequences obtained from lungfish transcriptomes finally settled the tetrapod relation: lungfish are the sister group of tetrapods (Amemiya, al., 2013).

The coelacanth genome sequence enabled testing the status of coelacanths as 'living fossil': The molecular evolutionary rate of protein coding genes is indeed significantly slower in coelacanth than that for most other vertebrate lineages (Amemiya, et al., 2013, Nikaido, et al., 2013). As inferred from the scaffold-level coelacanth genome assembly, karyotype evolution also seems to be relatively slow in coelacanth (Amemiya, et al., 2013), but this conclusion would need to be confirmed with a coelacanth chromonome. Transposable elements, in contrast, are abundant and have been recently active in coelacanth (Amemiya, et al., 2013, Chalopin, et al., 2013, Forconi et al., 2013, Nikaido, et al., 2013), which contrasts with the coelacanth's morphological stasis and slow rate of protein evolution.

The analysis of coelacanth genomes revealed their transitionary position among sarcopterygian vertebrates, showing genomic features of both 'fish' and tetrapods (Amemiya, et al., 2013, Nikaido, et al., 2013; coelacanth special issue of this journal). The coelacanth retained ancestral vertebrate genes that were lost in tetrapods and are thus present only in fish. Interestingly, genes lost in tetrapods but present coelacanth and teleost fish are involved in those structures that most dramatically changed during the water-to-land transition, such as paired appendages, which have different functions in water and on land, ears and eyes, which sense the environment different in water and in air, and kidneys, which have different stresses in dry air and water, suggesting that gene losses were an important component for the remodeling of the emerging tetrapod genome (Amemiya, et al., 2013). The most prominent examples for genes 'left in the water' are the *actinodin* genes (Amemiya, et al., 2013, Nikaido, et al., 2013) that encode structural components of fin folds in fish (Zhang et al., 2010).

In other aspects, however, the coelacanth genome shows similarities to tetrapods, for example in the constitution of its immune system (Boudinot et al., 2014, Saha et al., 2014) and olfactory gene repertoires (Nikaido, et al., 2013, Picone et al., 2013). Furthermore, several *cis*-regulatory elements involved in gene regulation during limb and lung development are unexpectedly conserved between tetrapods and coelacanth showing that important components of limb and lung gene regulatory networks predate the rise of tetrapods and evolved before the fin-to-limb transition and the evolution of terrestrial lungs (Amemiya, et al., 2013, Nikaido, et al., 2013).

Though a lot of additional analyses lie ahead, current studies of coelacanth genomes highlight several tetrapod genomic innovations that may have been involved in the adaptation to life on land. These include, for example, the emergence of putative gene regulatory elements around important developmental genes, including known limb development genes, and positive selection on components of the urea cycle (Amemiya, et al., 2013).

As critically endangered, livebearing, marine deep-water inhabitants, coelacanths are probably the worst imaginable fish to pursue functional investigations. Lungfishes have been informative, e.g., to study the evolution of walking mechanics (King et al., 2011) and of the pelvic apparatus (Boisvert et al., 2013). Furthermore, although mRNA *in situ* hybridization gene expression studies, e.g., during the development of lungfish digits homologs (Johanson et al., 2007) and lungfish dentition (Smith et al., 2009), have been performed, lungfish genome sizes and the challenges to obtain lungfish embryos make molecular studies very difficult. Functional studies of lobefin fish genes will thus have to rely on tests in other vertebrate species, as have been done for coelacanth conserved non-coding elements (CNEs) and coelacanth BACs in transgenic mice (Amemiya, et al., 2013).

4. Recent advances to study piscine gene functions in the postgenomic era

Regardless of the specific questions addressed using a particular fish model, any Evo-Devo study at some point will inevitably aim to turn insights from genetic, genomic, and transcriptomic analyses into functional hypotheses that ideally would be tested within the species under investigation and during the development of the trait. This requires the analysis of developmental processes *in vivo* and the study of spatio-temporal gene expression patterns with RNA *in situ* hybridization and/or immunohistochemistry. As described by examples above, these experiments may or may not be feasible depending on the specifics of the fish species, the regular availability of embryos (Fig. 1), feasibility of microinjections, working antibodies, etc. Functional analysis of gene evolution will require even more investment into developing species-specific tools. Chemical treatments with inhibitors and agonists of major developmental signaling pathways have been applied to a wide range of fish species because they can simply be added to the culture water. For example, mosquitofish (Ogino et al., 2004), bichir (Cuervo et al., 2012), and lungfish (Smith et al., 2009) have been treated in experiments with cyclopamine, an inhibitor of the hedgehog signaling pathway, to understand cell-cell signaling.

Functional assays, however, do not necessarily require doing functional experiments in the endogenous species itself. A seminal example is a study on cavefish albinism, showing that transfecting an Oca2 protein deficient, and therefore unpigmented murine pigment cell line with alleles of the *oca2* gene from normally pigmented surface Mexican tetra restores melanin synthesis, but transfection with alleles from albino cavefish populations fails to rescue pigmentation. This analysis provided functional evidence that sequence variation in the *oca2* gene, identified by QTL mapping as a major candidate, is indeed causing cavefish albinism (Protas, et al., 2006).

Functional tests will always be challenging in non-model fish species, even with sequenced genomes and other types of genomic resources. Thus, analogous to the study of the genetic basis of human evolution in 'humanized mice' (e.g., Prabhakar et al., 2008, Kamberov et al., 2013), we envision that in the future, zebrafish and medaka will be used as 'functional hubs' for Ostariophysi and Percomorpha (Fig. 1), respectively, to study the evolution of gene functions from a broad variety of fish. An example for such an interspecies approach is a platyfish-medaka tumor model, enabling functional tests that would not have been possible in platyfish itself because of its livebearing reproduction mode: the *Xmrk* tumor locus gene

from platyfish was integrated into the medaka genome to generate a stable transgenic medaka melanoma model (Schartl et al., 2010), which then enabled the *in piscine* study of tumorigenic events downstream of the constitutively active receptor tyrosine kinase encoded by platyfish's oncogene (Schartl et al., 2012, Liedtke et al., 2013). An instance for the use of zebrafish as a functional hub is the functional analysis of a candidate for the 'brown' locus in cavefish: Rescuing pigmentation in zebrafish embryos, in which Mc1r function was knocked-down with morpholino antisense oligo nucleotides, was successful with co-injection of surface but not cave *mc1r* RNAs, supporting that *mc1r* variation is responsible for the 'brown' phenotype (Gross, et al., 2009).

Functional tools for the analysis of gene actions have dramatically evolved for zebrafish and medaka within the past few years. Particularly, the toolbox for the analysis of *cis*-regulatory elements, which are at the heart of Evo-Devo research (Wray, 2007, Carroll, 2008), is rapidly transitioning. Putative *cis*-regulatory elements are often identified as conserved non-coding elements (CNEs) (e.g., Woolfe et al., 2005, Lee, et al., 2011, Lowe et al., 2011, Hiller, et al., 2013). The particularly high evolutionary turn-over rate of non-coding sequences in teleosts (Ravi and Venkatesh, 2008, Lee, et al., 2011), and the still insufficient genomic coverage of the rayfin lineage (Hiller, et al., 2013) makes the detection of CNEs in fish challenging. One solution to this problem comes with the application of ChIP-Seq, i.e., chromatin immunoprecipitation combined with high-throughput sequencing. Probing for DNA-protein interactions, ChIP-Seq can identify genome-wide transcription factor binding events or epigenetic marks indicative of enhancers and other types of gene regulatory elements. ChIP-Seq has assayed the activity of regulatory elements during zebrafish development (Aday et al., 2011, Bogdanovic et al., 2013). Because ChIP-Seq works independent of (although correlated with) sequence conservation, it enables to identification of newly evolved, lineage-specific *cis*-elements that would not turn up as conserved elements by comparing distant taxa.

The function of putative *cis*-elements are classically tested in transient or stable reporter construct assays in which the sequence of interest is cloned in front of a minimal promoter to drive a fluorescent protein in a spatio-temporal manner. These reporter constructs are then co-injected into 1–2 cell stage embryos of zebrafish or medaka with *Tol2* transposon RNA or meganuclease protein, respectively, which mediate integration of the construct into the host genome (e.g., Fisher et al., 2006, Grabher and Wittbrodt, 2007, Bessa et al., 2009). Similar transgenic methods should in principle also work in other fish species for which injectable, fertilized eggs in sufficient numbers are available, as shown for stickleback (Chan et al., 2010), some cichlids (Nakamura et al., 2008, Juntti et al., 2013), killifish (Valenzano et al., 2011), and others.

To study interactions of long-range enhancers and their target genes as well as to test more than just short, isolated, individual non-coding fragments, cross-species transgenesis in zebrafish or medaka with BAC clones from the species under investigation, usually covering ~150kb around the locus of interest, may be a method of choice. BAC transgenesis is now well established in zebrafish (Suster et al., 2011) and medaka (Nakamura, et al., 2008). An extreme example for the application of cross-vertebrate BAC transgenesis is a recent study

in which pufferfish BACs from the *hoxA* and *hoxD* clusters were transfected into mouse to investigate the regulatory basis of tetrapod digit origins (Woltering et al., 2014).

A common problem of these transgenic methods, however, is that the randomness of integration into the host genome makes results quite variable. The genomic environment of the integration site and its epigenetic state may influence expression of the reporter construct, a phenomenon known as 'positional effects' (Roberts et al., 2014). To overcome this problem, a new solution is on the horizon that will likely become a standard method in the fish field soon: PhiC31 integrase systems use landing sites integrated at well characterized spots of the host genome with low positional effects. These genomic *attP* landing sites recombine with the reporter construct's *attB* to accomplish site-directed transgenesis of the construct at a predefined genomic position in a repeatable manner (Hu et al., 2011). Various PhiC31 lines have been developed for medaka (Kirchmaier et al., 2013) and zebrafish (Mosimann et al., 2013, Roberts, et al., 2014), and the method should in principle even enable the integration of large, BAC-sized transgenes in fish (Kirchmaier, et al., 2013).

The breadth of mutants available for zebrafish and medaka further elevates their use as functional hubs for fish Evo-Devo. New methods for directed mutagenesis and genomic manipulation established for zebrafish, medaka, and a few other species (Fig. 1) that should be applicable to more fish systems come through the use of zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and most recently, the regularly interspaced short palindromic repeats (CRISPR)/Cas9 nuclease system (Ansai et al., 2013, Blackburn et al., 2013). The highly efficient CRISPR/Cas9 system uses a bacterial derived endonuclease directed by a short guide RNA to cleave DNA upstream of a protospacer adjacent motif. By inducing site-specific double-strand DNA breaks, the system can create indel mutations, targeted insertions, or substitutions, or, when multiple sites are targeted, larger scale deletions or inversions (Chang et al., 2013, Jao et al., 2013, Xiao et al., 2013). The flexibility and efficiency of these systems allow for the easy creation of loss-of-function mutations or the study of the loss of non-coding regulatory elements and the removal of enhancers. An example of such enhancer deletion comes from our own work (Fig. 5), the deletion of the well-studied *sonic hedgehog a* (*shha*) gene enhancer *ar-C* in zebrafish (Gehrig et al., 2009). Figure 5 shows that the CRISPR/Cas9 genome editing system ushers in a new phase in the analysis of gene regulatory networks in fish to study *in vivo* the effects of knocking out regulatory elements (and potentially also knocking in or switching alleles) on gene expression of the affected gene(s) and downstream effects in the network beyond just characterizing putative roles of *cis*-elements with reporter assay approaches.

5. Conclusions

With the current revolution in sequencing techniques, the genomic net covering fish evolution is becoming more and more fine-meshed, but is still leaky in view of the tremendous amount of biodiversity of rayfins and of teleost fish in particular. Orthology detection in teleost fish is more error-prone than in other vertebrate lineages because it needs to account for divergent evolution of teleost genomes and gene functions, which are aftermaths of the TGD, the genomic big bang at the base of the teleost lineage. Enabling

conserved synteny analyses, chromosome-level genome assemblies will be essential to help inform teleost gene orthologies. So far, genomic and transcriptomic methods have liberated multiple fish models from genomic ignorance, transforming them into a new model army for a comprehensive analysis of the genomic basis of evolution and development.

In combination with the general advantages of numerous fish systems to study the genetic underpinnings of vertebrate development, such as ease of husbandry, external development, high fertility, natural or chemically inducible embryo transparency, technical advances in the molecular and developmental analysis of fish gene functions suggest an auspicious future in which many fish model systems can enter a phase of functional investigations following the genetic, genomic and/or transcriptomic characterization of their evolutionary diversity. For species in which direct functional testing *in vivo* is not practical, testing their gene functions in zebrafish or medaka may become the method of choice.

Finally, as outreach and societal impact become increasingly important criteria for research funding, we believe that – because everybody knows (and likes!) fish from fishing, fishkeeping, diving, and the dinner table – the 'Fish World' is also an excellent motivating educational device to teach systematics, evolution, developmental biology, morphology, physiology, and ecology to the general public.

Acknowledgments

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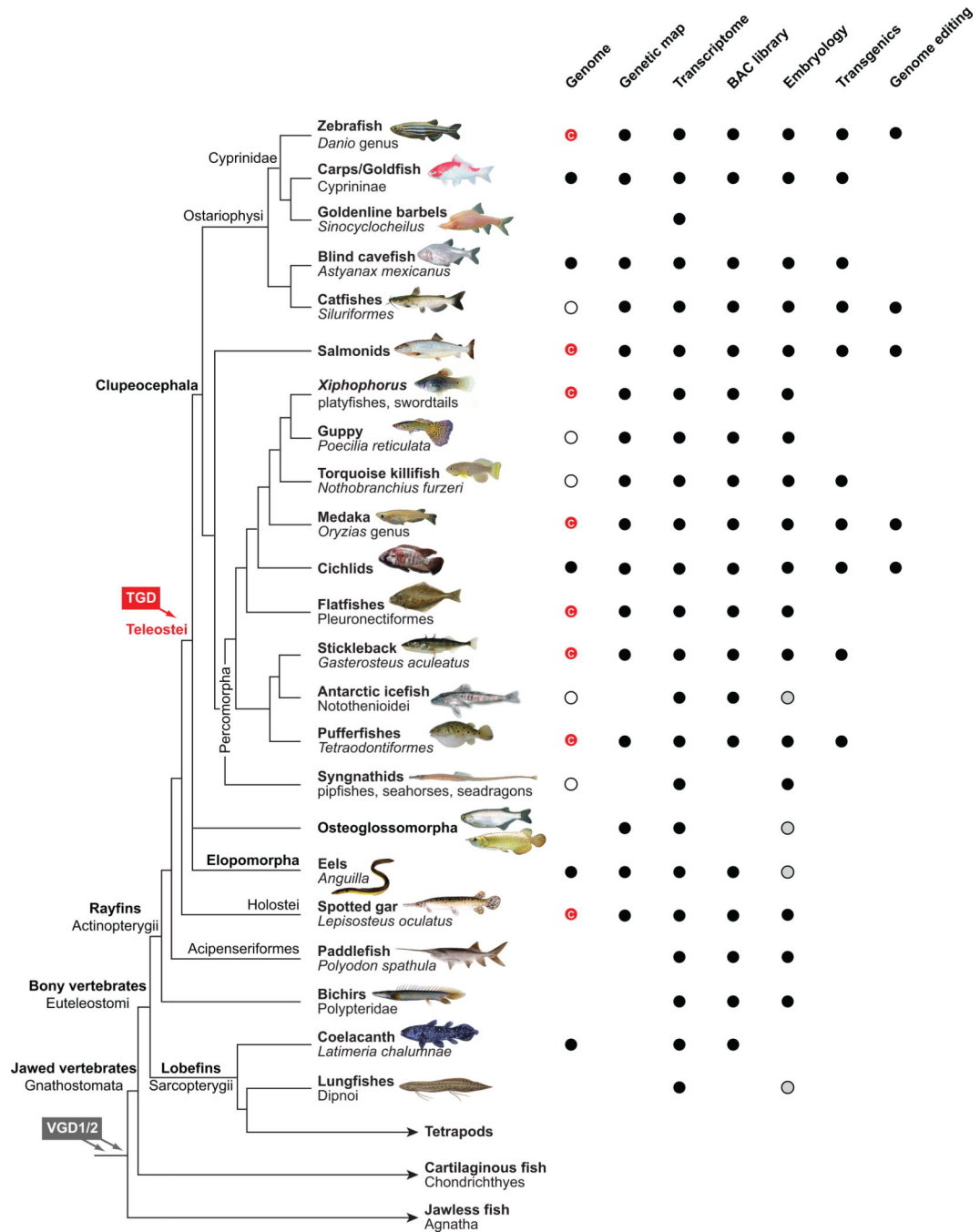
Fig. 1. Bony vertebrate fish models for evolutionary developmental genomics (Evo-Devo-Geno) Phylogenetic relationships are shown to the left, available genomic resources (genome assembly; genetic maps; transcriptomes; BAC libraries) and functional tools (embryology: described and available embryonic developmental stages; transgenic and genome editing methods) to the right. See Table 1 for Evo-Devo-Geno research questions for each model. Genome circles: open circle, ongoing genome sequencing project(s); red circle with 'C', chromosome-level reference genome (chromonome) available. Embryology circles: grey circle, spawns occasionally available, but technically highly demanding. VGD1/2: vertebrate

genome duplications 1 and 2; TGD: teleost genome duplication. Tree topology is based on Nelson (2006), Near, et al. (2012b), and Betancur, et al. (2013).
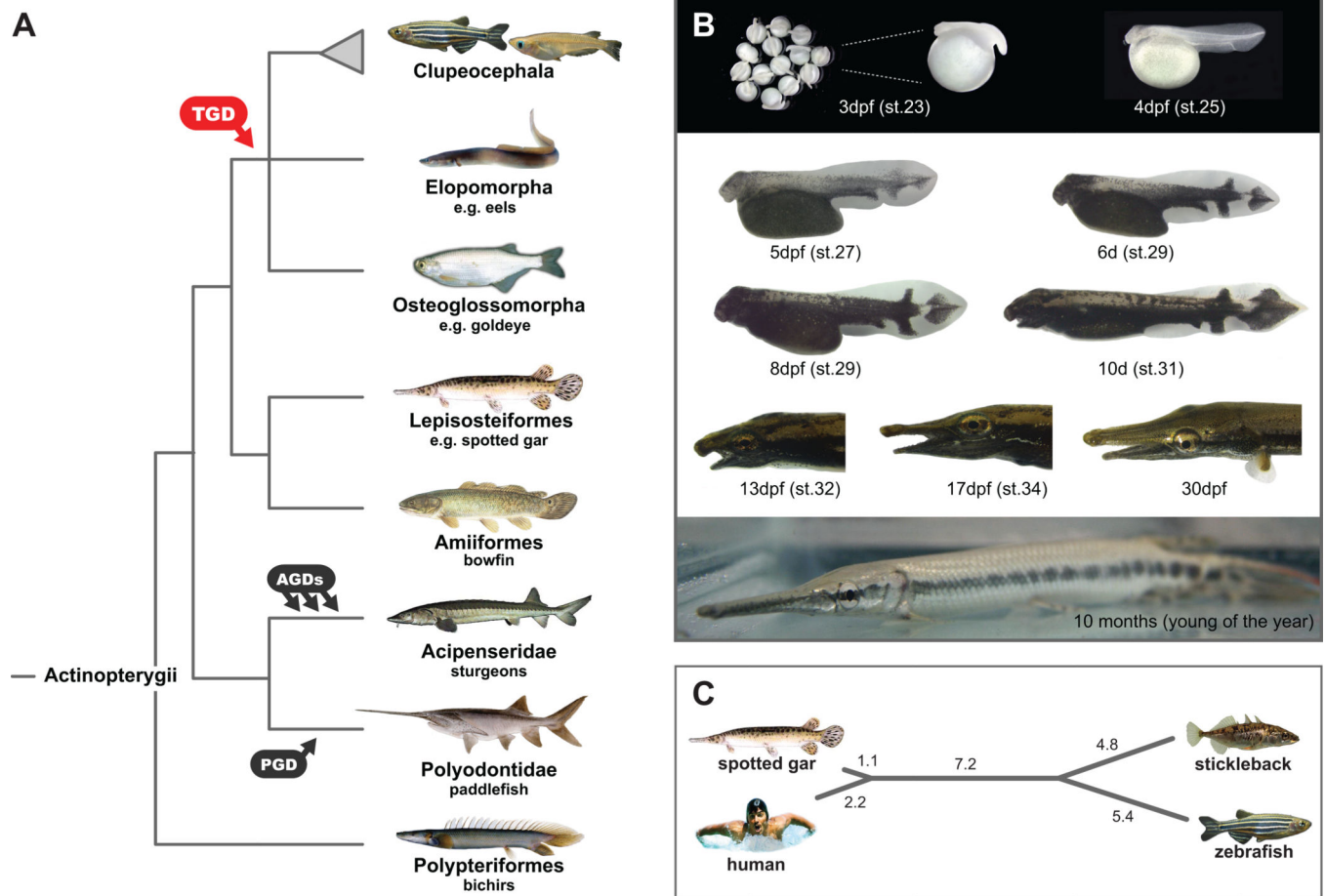
Fig. 2. Rayfin phylogenetic relationships and the spotted gar model

A) Phylogeny of major living rayfin lineages. TGD: teleost genome duplication; AGDs: acipenserid (sturgeon) genome duplications; PGD: paddlefish genome duplication. B) Development of the spotted gar (*Lepisosteus oculatus*) in days post fertilization (dpf). Assignment of developmental stages (st.) follows Long and Ballard (2001). C) Comparison of conserved syntenies between teleosts (stickleback and zebrafish) with respect to gar and human (adapted from Amores et al., 2011). Branch lengths are proportional to conserved syntenies per 100 million years of divergence time. Branch lengths between non-teleosts (gar and Mark Spitz) are much shorter than lengths between either of these two species and teleosts, suggesting massive chromosomal rearrangements in teleosts after their divergence from the gar lineage.
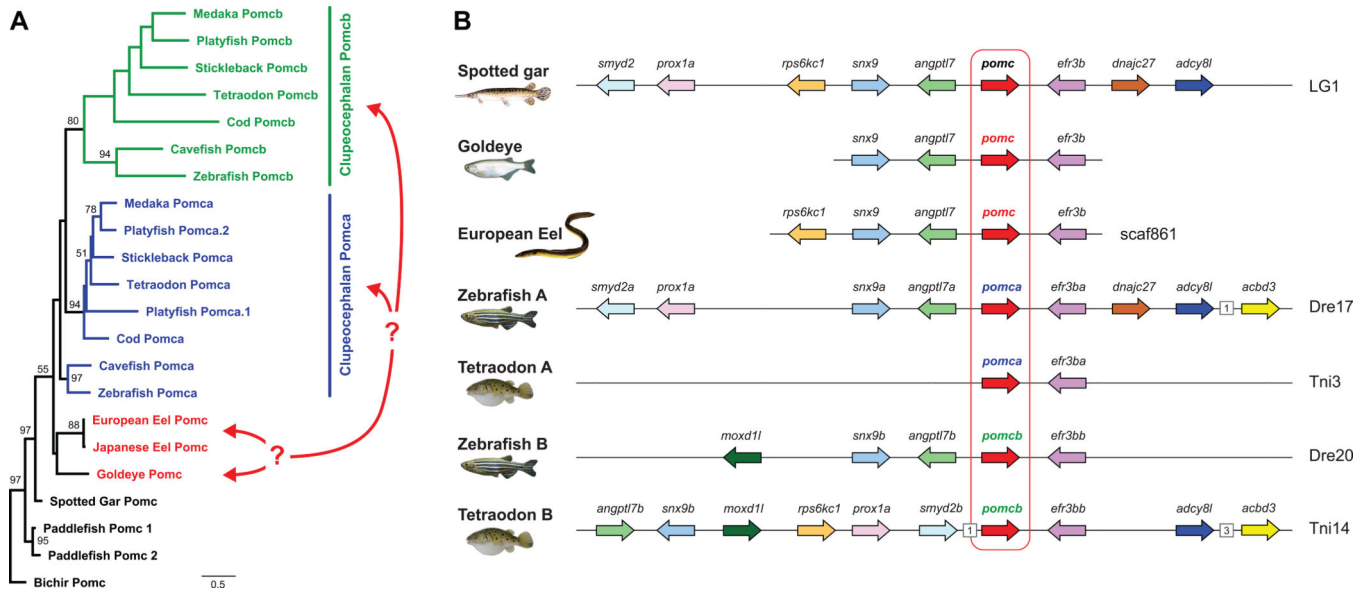
Fig. 3. Evolution of the rayfin proopiomelanocortin (*pomc*) gene

A) Maximum likelihood phylogeny (JTT+I+G+F model) of full-length Pomc proteins in rayfins rooted with bichir Pomc. Node values indicate support from 100 bootstrap replications, showing bootstrap values >50% only. Clupeocephalans teleosts retained both TGD *pomc* paralogs; their assignment to *a*- and *b*-paralogs follows de Souza et al. (2005) (monophyly of clupeocephalan Pomca is not supported in the current tree). In eels (elopomorphs) only a single *pomc* gene has been found and for goldeye (osteoglossomorphs), we have found so far only one *pomc* gene (but we cannot exclude the presence of the second TGD paralogs yet). The relationship of these *pomc* singletons among each other as well as to clupeocephalan *pomca* and *pomcb* remain unresolved (indicated by '?'). Two *pomc* genes are present in paddlefish, most likely as result of the paddlefish-specific polyploidization; two *pomca* paralogs are present in platyfish and other percomorphs as result of an additional gene duplication (Harris et al., 2014). B) Conserved syntenies of rayfin *pomc* gene regions. Neighboring genes of the single *pomc* gene in goldeye and eel are conserved with spotted gar and both zebrafish *pomc* paralogons, which makes it impossible to make an *a/b*-orthology call, which would require more large-scale chromosomal assemblies from the basal teleosts. Tetraodon *pomc* paralogons are more rearranged than those from zebrafish. Boxed numbers indicate intervening regions that do not contribute to conserved synteny.

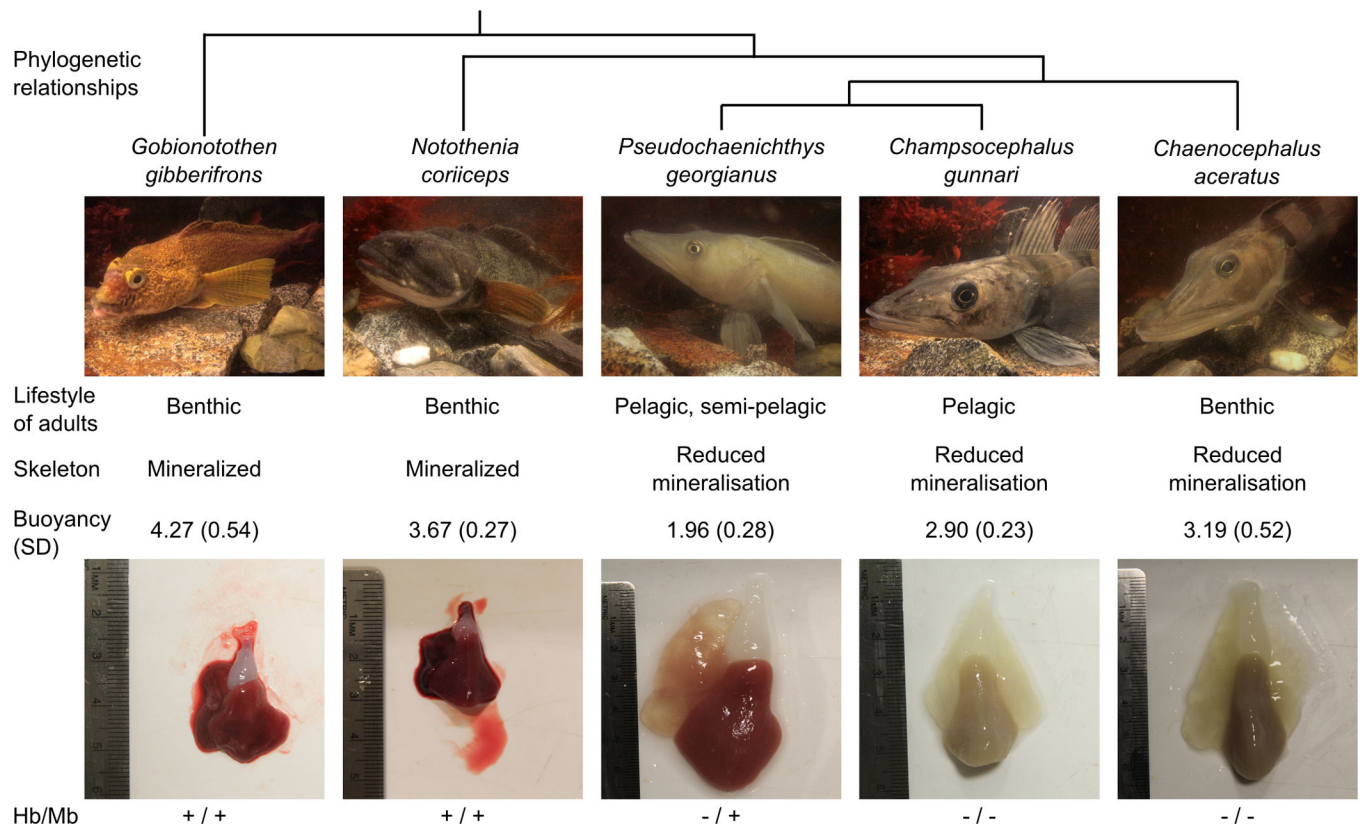|	*Gobionotothen gibberifrons*	*Notothenia coriiceps*	*Pseudochaenichthys georgianus*	*Champsocephalus gunnari*	*Chaenocephalus aceratus*
Phylogenetic relationships					
Lifestyle of adults	Benthic	Benthic	Pelagic, semi-pelagic	Pelagic	Benthic
Skeleton	Mineralized	Mineralized	Reduced mineralisation	Reduced mineralisation	Reduced mineralisation
Buoyancy (SD)	4.27 (0.54)	3.67 (0.27)	1.96 (0.28)	2.90 (0.23)	3.19 (0.52)
Hb/Mb	+ / +	+ / +	- / +	- / -	- / -

Fig. 4. Diversity of ecological and physiological features in notothenioids
Phylogenetic relationships and the buoyancy data are from Near et al. (2012a) and information on adult lifestyles is reviewed in Rutschmann et al. (2011). A dissected heart is shown for each species and presence (+) and absence (−) of hemoglobin and myoglobin (Hb/Mb) is indicated as well.
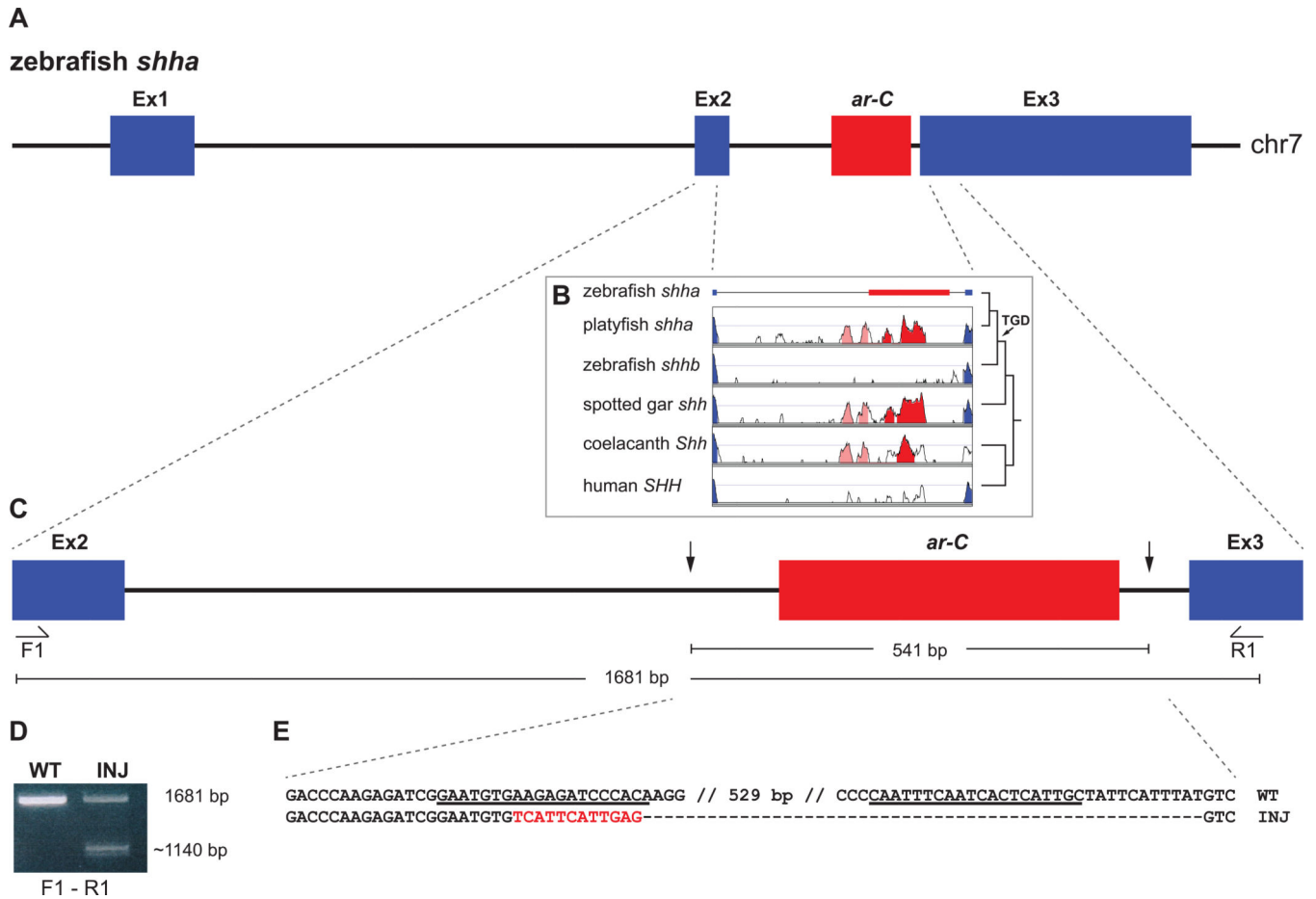
Fig. 5. Deletion of the *shha ar-C* enhancer using the CRISPR/Cas9 system in zebrafish
A) The zebrafish *ar-C* enhancer is located between exon (Ex) 2 and 3 of the *sonic hedgehog a* (*shha*) gene on chromosome (chr) 7. B) VISTA plot showing sequence conservation of zebrafish *shha* intron 2 (reference sequence) to other species. TGD: teleost genome duplication. The location of the *ar-C* region was defined by Gehrig et al. (2009). C) The CRISPR/Cas9 system was employed to remove the enhancer by targeting two sites flanking the region. The locations of the targeted regions are indicated with arrows; primers (F1 and R1) were used for PCR detection of induced deletions. D) Deletions of the expected size in injected embryos (INJ) were confirmed by PCR, and were not present in uninjected embryos (WT). E) A representative example of a sequenced allele in an injected embryo verified the removal of the *ar-C* enhancer with the sequence of the *shha* coding sequence remaining intact. The target region is underlined and the protospacer adjacent motif site is indicated in bold. Deletions are indicated with dashes while inserted nucleotides are indicated in red. The experiment shows that the targeted deletion of *cis*-regulatory elements using the CRISPR/Cas9 system is a useful new tool for the Evo-Devo-Geno toolbox in fish.

Table 1
Research questions of bony vertebrate fish model systems

See Figure 1 for phylogenetic relationships, available genomic resources, and functional tools.

Lineage	Exemplary Evo-Devo-Geno research questions	Further reading
Danio complex	pigmentation, dwarfism, novel morphological structures, sex determination, barbels	section 3.3; Howe, et al., 2013b
Carps/Goldfish	fin structures, scale patterns, pigmentation, domestication, polyploidy	section 3.3; Henkel, et al., 2012c
Goldenline barbels	cave phenotypes, eye loss, albinism, brain structure, sensory organs, polyploidy	section 3.4
Blind cavefish	cave phenotypes, eye loss, albinism, brain structure, craniofacial structures, sensory organs	section 3.4
Catfishes	skeletal structures, pigmentation, mimicry, polyploidy (*Corydoras*)	Alexandrou et al., 2011, Jiang et al., 2013
Salmonids	*hox* clusters, polyploidy, skeletal structures, sex determination, evolution of anadromous features	Berthelot et al., 2014; Davidson, et al., 2010
Xiphophorus complex	pigmentation, fin structures, viviparity, sex-linked traits, sex determination, behavior, hybridization	section 3.5; Schartl, et al., 2013
Guppy	pigmentation, fin structures, viviparity, sex-linked traits, sex determination, behavior	section 3.5
Torquoise killifish	aging, pigmentation	Valenzano et al., 2009, Petzold et al., 2013
Oryzias complex	pigmentation, fin structures, sex determination, craniofacial traits	Kasahara, et al., 2007, Takeda and Shimada, 2010, Spivakov et al., 2014
Cichlids	craniofacial adaptations, pigmentation, body shape, sex determination, behavior, adaptive radiation	Kocher, 2004, Salzburger, 2009, Fan et al., 2012, Santos and Salzburger, 2012
Flatfishes	left-right asymmetry, metamorphosis, pigmentation, sex determination/reversal	Cerda and Manchado, 2013, Chen, et al., 2014, Shao et al., 2014
Stickleback	skeletal traits, craniofacial structures, sex determination, pigmentation, behavior	Peichel, 2005, Cresko et al., 2007, Hohenlohe et al., 2010, Jones, et al., 2012
Antarctic icefish	bone development, loss of hemoproteins, cold adaptation, heat shock, adaptive radiation	section 3.6
Pufferfishes	body plan reduction, genome size reduction, sex determination	Aparicio, et al., 2002, Amores et al., 2004, Jaillon, et al., 2004
Sygnathids	male pregnancy, body plan changes, fin structures	Stolting and Wilson, 2007, Mobley et al., 2011
Osteoglosso-morpha	genome evolution, *hox* clusters, air breathing, electric organs (mormyrids)	section 3.2
Eels	genome evolution, *hox* clusters, fin loss, body elongation	section 3.2; Henkel, et al., 2012a, Henkel, et al., 2012b
Spotted gar	genome evolution, fin structures, air breathing, teleost innovations (outgroup), tetrapod innovations (outgroup)	section 3.1
Paddlefish	tetrapod fin-to-limb transition (outgroup), fin structures, polyploidy, *hox* clusters, electroreception	section 3.1
Bichirs	fin structures, early embryonic development, *hox* clusters	section 3.1
Coelacanth	tetrapod fin-to-limb transition and other tetrapod innovations (outgroup), 'living fossil'	section 3.7; Amemiya, et al., 2013, Nikaido, et al., 2013
Lungfishes	tetrapod fin-to-limb transition and other tetrapod innovations (outgroup), dentition, genome evolution	section 3.7