Int. J. Dev. Biol. 58: 863-871 (2014)
doi: 10.1387/ijdb.140316kk

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Reptile genomes open the frontier for comparative analysis of amniote development and regeneration

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ABSTRACT Developmental genetic studies of vertebrates have focused primarily on zebrafish, frog and mouse models, which have clear application to medicine and well-developed genomic resources. In contrast, reptiles represent the most diverse amniote group, but have only recently begun to gather the attention of genome sequencing efforts. Extant reptilian groups last shared a common ancestor ~280 million years ago and include lepidosaurs, turtles and crocodilians. This phylogenetic diversity is reflected in great morphological and behavioral diversity capturing the attention of biologists interested in mechanisms regulating developmental processes such as somitogenesis and spinal patterning, regeneration, the evolution of "snake-like" morphology, the formation of the unique turtle shell, and the convergent evolution of the four-chambered heart shared by mammals and archosaurs. The complete genome of the first non-avian reptile, the green anole lizard, was published in 2011 and has provided insights into the origin and evolution of amniotes. Since then, the genomes of multiple snakes, turtles, and crocodilians have also been completed. Here we will review the current diversity of available reptile genomes, with an emphasis on their evolutionary relationships, and will highlight how these genomes have and will continue to facilitate research in developmental and regenerative biology.

KEY WORDS: *reptile, genomics, gene expression, somitogenesis, regeneration*

Introduction

A major goal of developmental genomics is to understand the genetic mechanisms underlying vertebrate patterning and differentiation. The most successful and diverse group of modern land-adapted vertebrates are amniotes, and they display a wide array of forms, from parakeets to people to pythons. This diversity comes with the opportunity to learn about shared and divergent pathways regulating development during the evolution of the vertebrate body plan. Much has been learned, through comparisons of amniote (chicken and mouse) and anamniote (zebrafish and *Xenopus*) developmental and genomic models, about molecular mechanisms that underlie important pathways. The human genome project brought with it the initial promise that we would one day understand the true origins of human genes and genetic disorders (Lander *et al.*, 2001) and the more recent advent of next-generation sequencing technologies has yielded assembled genome representatives for most mammalian orders (Chinwalla *et al.*, 2002; Lindblad-Toh *et al.*, 2005; Liu *et al.*, 2009; Mikkelsen *et al.*, 2007; Wade *et al.*, 2009; Warren *et al.*, 2008; Zhang *et al.*, 2013) in addition to 28 avian genomes (Table 1; Zhang *et al.,* 2014). These sequences are freely available to the scientific community as the foundation for developmental studies. For instance, one could easily navigate to the University of California, Santa Cruz (UCSC) Genome Browser (available at http://genome.ucsc.edu/) and access the complete genomes of 48 mammals and five of the aforementioned birds. Next-generation sequencing has facilitated genomic studies of non-traditional model organisms at a cheaper cost, and the genomes of many more vertebrate species are being sequenced, contributing to the Genome 10K project (Genome 10K Community of Scientists, 2009). Of the more than 30,000 living amniote species, almost 10,000 are reptiles, yet there have been relatively few genomic resources available for non-avian reptiles until only very recently. This is despite the fact that non-avian reptiles contain far more diversity than mammals and birds in many aspects of development and physiology. Here we will review the phylogenetic diversity of currently available reptile genomes, and discuss how they have contributed to the knowledge of vertebrate developmental biology. Also, we will review current and potential avenues of research that are shedding light on comparative studies

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Accepted: 19 December 2014

ISSN: Online 1696-3547, Print 0214-6282

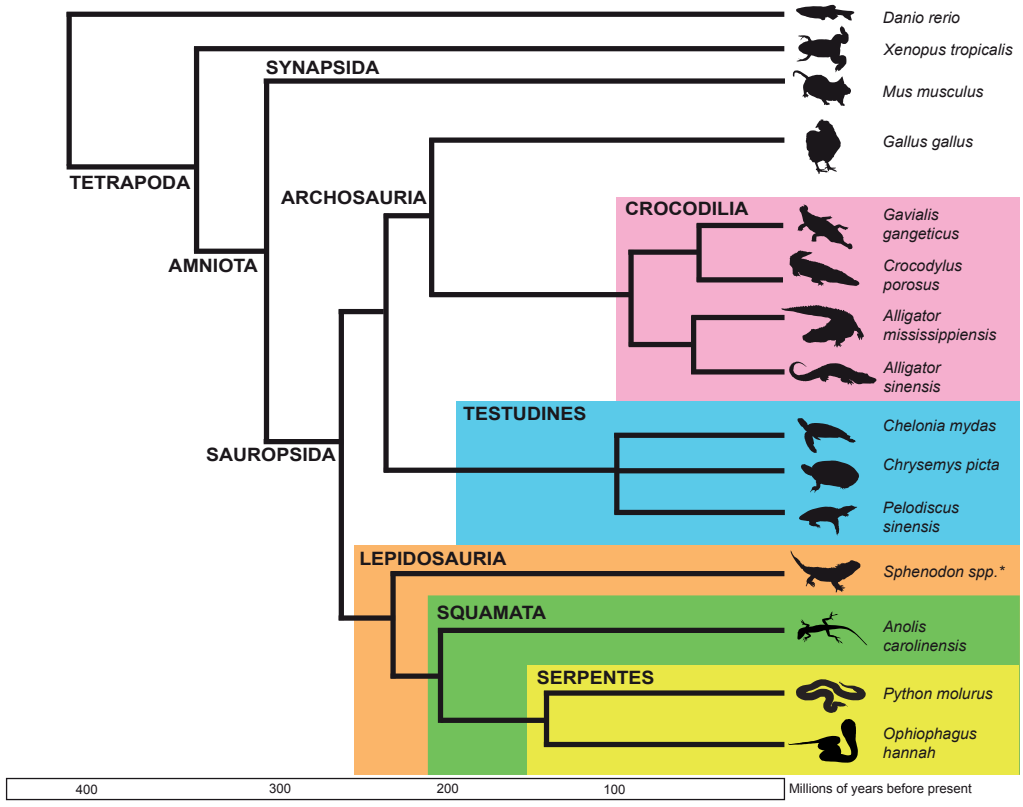

Fig. 1. Vertebrate phylogeny highlighting reptiles with complete genome sequences. *Select model organisms are included as outgroups* (Danio rerio, Xenopus tropicalis, Mus musculus). *Names of key vertebrate clades are given at the appropriate nodes in the phylogeny (Tetrapoda, Amniota, Sauropsida, Archosauria). The only remaining extant lineage of the Synapsida is the mammals. The evolutionary relationships of the modern reptilian lineages (Lepidosauria, Testudines and Crocodilia) and the species for which there are complete genomes available (as of November 2014) are featured. Birds (represented here by* Gallus gallus*) are in the Archosauria, see Table 1 for avian genome resources. *There is not currently a complete genome available for* Sphenodon*, but it is included in the phylogeny to bring attention to the phylogenetic position of tuataras.*

of regenerative capacity. Here, we will use the term "reptiles" in the historical sense to include the following: Testudines, or all living turtles; Crocodilia, or alligators, caimans, gharials and crocodiles; and Lepiodosauria, or all lizards, snakes, amphisbaenians (or all squamates) and the tuatara. For each of these groups, we will review their unique sets of adaptations and phenotypes, and how genome-sequencing efforts have facilitated work in that area.

The traditional class "Reptilia" refers to the living ectothermic amniotes. Although they share with amphibians several aspects of lifestyle, behavior, ecology and a whole field of study known as herpetology, as amniotes reptiles are more closely related to mammals and birds. After radiating into terrestrial environments during the Carboniferous around 320-310 million years ago (Donoghue, Benton, 2007; Pyron, 2010) (Fig. 1), amniotes split into two recognized groups based on cranial morphology: synapsids and sauropsids (Benton, 2005). The synapsids include all mammals as well as many extinct lineages of "mammal-like reptiles" from the Permian and Mesozoic Eras (i.e., pelycosaurs, therapsids and cynodonts). Synapsids reached the peak of their diversity in the Permian, and most lineages disappeared at the Permian-Triassic boundary extinction event; the remaining extant synapsids constitute class "Mammalia". While their ancestors were similar in many respects to modern reptiles, modern mammals differ a great deal from reptiles in several important traits such as endothermy, mammary glands, fur, and the eutherian placenta.

The second amniote group is the sauropsids, which includes all living reptiles and birds, and originated 250-280 million years ago (Fig. 1). Sauropsids survived the Permian-Triassic extinction and diversified to dominate terrestrial and marine environments throughout the Mesozoic Era. The evolutionary history of sauropsids includes a Lepidosauria branch and a branch containing the order Testudines and the Archosauria, which includes birds and crocodiles. While the surviving modern reptiles constitute "saurian" reptiles, birds evolved from dinosaurs (Gauthier 1986; Brusatte *et al.*, 2010) and so are also sauropsids, albeit with a set of highly derived set of "non-reptilian" constraints such as endothermy, feathers, and flight. A recent integration of developmental and paleontological evidence has clarified patterns of loss, fusion and re-evolution of wrist features that were integral to the early evolution of flight in bird-like dinosaurs and their avian descendants (Botelho *et al.,* 2014). When focusing on the "reptilian" sauropisds, the times to the most recent common ancestors of Testudines, Archosauria, and Lepidosauria are on average much older (~240 million years) than those between the modern placental mammalian orders (~100 million years) and even between placental mammals and monotremes (~200 million years ago) (Donoghue, Benton, 2007; Pyron, 2010), making the sequence of divergence between the sauropsid orders – and thus the ancestral states of various developmental milestones and divergent phenotypes – a controversial subject. One major area of disagreement has been the placement of turtles on phylogenetic trees. Turtles have a unique body plan, the most obvious trait being the shell, as well as a lack of temporal fenestrae in the turtle skull, differing greatly from the skulls of synapsids and other sauropsids that do contain fenestrae. Earlier studies based on anatomy and paleontology placed turtles in the sister lineage to all other amniotes ("Parareptilia") (Benton, 2005). Some more recent genetic work has suggested that turtles form a clade with lepidosaurs (Lyson *et al.*, 2012). However, the majority of genomic evidence supports a turtle-archosaur clade (Crawford *et al.*, 2012; Crawford *et al.*, 2014; Shaffer *et al.*, 2013; Wang *et al.*, 2013) and it is likely that the loss of temporal fenestrae was

a signature trait in the early evolution of turtles (Kuratani *et al.*, 2011) and we adopt this approach in this review (Fig. 1).

Genomic resources for Reptiles

Complete genomes for Lepidosauria

The first reptile to have a complete genome sequence was a lepidosaur, the green anole lizard (*Anolis carolinensis*), which was made available by the Broad Institute in 2007 and published in 2011 (Table 1; Alföldi *et al.*, 2011). It was mainly chosen to bridge the phylogenetic gap between chicken and human for comparative genomic studies in order to understand the origin of human genes (Janes *et al.*, 2010), and its initial analysis yielded important insights to the evolution of amniote genomes (Alföldi *et al.*, 2011). For instance, very few chromosomal rearrangements have occurred since *A. carolinensis* diverged from chicken ~280 million years ago, and there is a high degree of synteny conservation. In addition, the lack of isochores in the green anole genome suggested for the first time that GC content may be less integral to genomic integrity that previously thought (Fujita *et al.*, 2011).

Since the release of the green anole genome, the genomes of two other lepidosaurs have been made available. The first was the Burmese python (*Python molurus bivittatus*), which was announced in 2011 (Castoe *et al.*, 2011) and its complete genome was published in 2013 (Castoe *et al.*, 2013). The analysis of the python genome highlighted large variation in gene expression associated with changes in organ size and metabolism due to the feast-and-famine lifestyle of snakes. Two other lepidosaur genomes belong to snakes as well: the king cobra *(Ophiophagus hannah)* was sequenced to better understand the regulatory components and evolutionary origins of the complex venom system (Vonk *et al.,* 2013), while the first analysis of the speckled rattlesnake *(Crotalus mitchelli)* draft genome focused on multiple episodes of endogenous viral element integration (Gilbert *et al.,* 2014).

Complete genomes for Testudines

The first published turtle genome was that of the western painted turtle (*Chrysemys picta*), and its initial analysis focused on the molecular bases of tooth loss, immunity, longevity and adaptations for anoxic conditions (Shaffer *et al.*, 2013). Following the release of the painted turtle genome was an analysis of two other turtle genomes, the green sea turtle (*Chelonia mydas*) and the Chinese softshell turtle (*Pelodiscus sinensis*), which used gene expression analyses to understand common and divergent developmental patterns across amniotes with a focus on the unique turtle shell (Wang *et al.*, 2013). Combined, these resources provide a firm foundation for future studies on development in turtles, which significantly diverges from the ancestral amniote morphology.

Complete genomes for Crocodilia

While they traditionally have been placed in the class "Reptilia", crocodilians are archosaurian reptiles that share common ancestry within modern birds (Brusatte *et al.*, 2010), and therefore have the most promise for understanding genomic and developmental

TABLE 1

SAUROPSIDS, INCLUDING REPTILES AND BIRDS, WITH AVAILABLE COMPLETE GENOME SEQUENCES

Scientific name	Common name	Order	Family	Year reported	DOI
LEPIDOSAURIA					
Anolis carolinensis	Green anole	Squamata	Dactyloidae	Data released 2007	10.1038/nature10390
Python molurus	Burmese python	Squamata	Pythonidae	2011	10.1186/gb-2011-12-7-406
Ophiophagus hannah	King cobra	Squamata	Elapidae	2013	10.1073/pnas.1314702110
TESTUDINES					
Chrysemys picta bellii	Western painted turtle	Testudines	Emydidae	2013	10.1186/gb-2013-14-3-r28
Pelodiscus sinensis	Chinese soft-shelled turtle	Testudines	Trionychidae	2013	10.1038/ng.2615
Chelonia mydas	Green sea turtle	Testudines	Chelonidae	2013	10.1038/ng.2615
CROCODILIA					
Gavialis gangeticus	Indian gharial	Crocodilia	Gavialidae	2012	10.1186/gb-2012-13-1-415
Crocodylus porosus	Saltwater crocodile	Crocodilia	Crocodylidae	2012	10.1186/gb-2012-13-1-415
Alligator mississippiensis	American alligator	Crocodilia	Alligatoridae	2012	10.1186/gb-2012-13-1-415
Alligator sinensis	Chinese alligator	Crocodilia	Alligatoridae	2013	10.1038/cr.2013.104
AVES					
Anas platyrhynchos	Mallard duck	Anseriformes	Anatidae	2013	10.1038/ng.2657
Falco cherrug	Saker falcon	Falconiformes	Falconidae	2013	10.1038/ng.2588
Falco peregrinus	Peregrine falcon	Falconiformes	Falconidae	2013	10.1038/ng.2588
Gallus gallus	Chicken	Galliformes	Phaisanidae	2004	10.1038/nature03154
Meleagris gallopavo	Turkey	Galliformes	Phaisanidae	2010	10.1371/journal.pbio.1000475
Tetrao tetrix	Black grouse	Galliformes	Phaisanidae	2014	10.1186/1471-2164-15-180
Pseudopodoces humilis	Tibetian ground tit	Passeriformes	Paridae	2013	10.1186/gb-2013-14-3-r29
Taeniopygia guttata	Zebra finch	Passeriformes	Estrildidae	2010	10.1038/nature08819
Geospiza fortis	Medium ground-finch	Passeriformes	Thraupidae	Data released 2012	10.5524/100040
Geospiza magnirostris	Large ground-finch	Passeriformes	Thraupidae	2013	10.1186/1471-2164-14-95
Ficedula albicollis	Collared flycatcher	Passeriformes	Muscicapidae	2012	10.1038/nature11584
Ficedula hypoleuca	Pied flycatcher	Passeriformes	Muscicapidae	2012	10.1038/nature11584
Amazona vittata	Puerto Rican parrot	Psittaciformes	Psittacidae	2012	10.1186/2047-217X-1-14
Melopsittacus undulatus	Budgerigar	Psittaciformes	Psittaculidae	2011	10.1186/2047-217X-3-11

evolution in the avian lineage (Fig. 1). The International Crocodilian Genomes Working Group (www.crocgenomes.org) published preliminary assemblies for the American alligator (*Alligator mississippiensis*), the saltwater crocodile (*Crocodylus porosus*) and the Indian gharial (*Gavialis gangeticus*), and provide transcriptome data from various tissues to aid in annotation, as well as bacterial artificial chromosome (BAC) sequences to improve assemblies (St John *et al.*, 2012). Final assemblies and annotations for these species, as well as a robust evolutionary analysis shedding light on the ancestral evolution of the archosaur lineage was subsequently produced by the same group (Green *et al.,* 2014). The genome of a fourth crocodilian species, the Chinese alligator (*Alligator sinensis*), which is listed as critically endangered on the International Union for Conservation of Nature (IUCN) Red List (http://www.iucnredlist.org/details/867/0) was sequenced and analyzed in the context of molecular adaptations to long-period diving behavior. Lineage-specific expansions of genes related to the robust crocodilian immune system were found in the Chinese alligator genome (Wan *et al.*, 2013).

Transcriptomic resources for Reptiles

While the complete sequencing of a reference genome will certainly facilitate studies of genes and their expression and can shed light on developmental processes, the *de novo* sequencing and assembly of transcriptomes by way of next-generation sequencing technologies (i.e., RNA-Seq) in the absence of a reference genome has also been useful (Gibbons *et al.*, 2009). To date, several transcriptome resources have been developed for reptiles lacking a complete genome, including the western terrestrial garter snake (*Thamnophis elegans*) (Schwartz *et al.*, 2010) and the common chameleon (*Chamaeleo chamaeleon*) (Bar-Yaacov *et al.*, 2013). Complete brain transcriptomes have been generated for the Nile crocodile (*Crocodylus niloticus*), the corn snake (*Pantherophis gutattus*), the bearded dragon (*Pogona vitticeps*) and the red-eared slider turtle (*Trachemys scripta*) (Tzika *et al.*, 2011) (available at www.reptilian-transcriptomes.org), and the vomeronasal organ transcriptome has been generated for the corn snake (Brykczynska *et al.*, 2013).

A particularly valuable resource will be the transcriptome of the tuatara (*Sphenodon punctatus*) (Miller *et al.*, 2012), which is a non-squamate lepidosaur (Fig. 1). *Sphenodon* is the surviving genus of the order Rhyncocephalia, which had a global distribution until the late Cretaceous (65-80 million years ago) (Apesteguía, Novas, 2003). The range of *Sphenodon* today is limited to a few small islands in New Zealand. Until there is a complete genome, the tuatara transcriptome will facilitate future research in various avenues of genomic evolution and conservation of reptiles. As complete genomes are now available for all major groups of reptiles (lepidosaurs, turtles and archosaurs), these transcriptomes can be easily mapped to their nearest relatives (*Sphenodon* to *Anolis*, for example), and can help shed light on the diversity and numbers of reptilian transcripts and how they differ from current model amniotes representing mammals and birds.

Evolution of gene families in reptiles

Based on prediction and homology alone, the green anole genome was initially reported to contain 17,472 protein-coding genes that were largely predicted through *ab initio* efforts (Alföldi *et al.*, 2011). A subsequent transcriptome-based annotation increased the gene number to 22,962 (Eckalbar *et al.*, 2013), which is comparable to other amniotes. In comparison, 25,385 genes were annotated in the python genome (Castoe *et al.*, 2013), although only 68% of these contained a protein domain. 21,796 protein-coding genes were found in the painted turtle genome (Shaffer *et al.*, 2013), and ~22,200 were reported in the Chinese alligator (Wan *et al.*, 2013). The initial prediction for the chicken genome was that it contained between 20,000 and 23,000 protein coding genes (International Chicken Genome Sequencing Consortium, 2004). The mouse genome (GRCm38, accessed from www.ensmbl.org) contains 22,592 protein coding genes, and the number of protein coding genes found in the human genome by the ENCODE project was 20,687 (The ENCODE Project Consortium, 2012), although recent work shows that this number for humans could be reduced to less than 20,000 (Ezkurdia *et al.*, 2014). This suggests that the total number of expected genes in any amniote genome should be in the range of 20,000 genes. Nonetheless, gene evolution across amniotes has been dynamic with considerable gene family loss and/or expansion since the time of divergence between the living vertebrates. For instance, the green anole lizard genome contained 3,994 protein coding genes with one-to-one orthologues in human, mouse, dog, opossum, platypus, chicken, zebra finch and pufferfish (Alföldi *et al.*, 2011), which is considerably less than the total number of predicted genes for each of these genomes and suggests a high degree of gene duplication and loss during the evolution of these lineages.

The differences in various gene family expansions between reptiles and mammals are substantial, and have been linked to particular adaptations that are unique to each lineage. For instance, 11 opsin gene families were present in the green anole lizard genome as well as several species of invertebrate, fish and frog, but are absent in mammals (Alföldi *et al.*, 2011) and this was related to the superior color vision in lizards when compared to most mammals. In addition, the green anole lizard genome featured significant duplications and expansion of several egg protein gene families, with an elevated rate of molecular evolution that indicates episodic positive selection and bouts of adaptation. Vivipary, or the birth of live young, evolved early and often during the diversification of squamates (115 times versus 140 in all vertebrates) (Pyron, Burbrink, 2014), with frequent reversions to ovipary which would require many changes in egg-laying at the molecular level during sauropsid evolution. Indeed, out of the 276 protein-encoding genes expressed in the eggs of *A. carolinensis*, only 50 orthologues were confirmed in chicken, suggesting high turnover. Significant expansion of olfactory receptor families were found in the soft shell turtle genome, including 1,137 intact and possibly functional genes which is an amount similar to what is found in most mammals (Wang *et al.*, 2013). Other examples of reptile-specific and functionally-related gene family expansion are the venom proteins in snakes, as revealed by comparisons between the python and king cobra genomes (Castoe *et al.*, 2011; Vonk *et al.*, 2013), and the contrasting evolutionary patterns of vomeronasal receptor repertoires that were observed between mammals and reptiles (Brykczynska *et al.*, 2013).

Genome vs. transcriptome based expression studies in reptiles

Though genomes are continually being released, there are many species of interest for which a genome is not available. Mapping

RNA-Seq reads to the reference genome of the same species remains the "gold standard" for gene expression studies (Fig. 2A) (Guttman *et al.*, 2010; Trapnell *et al.*, 2012); however, for those species without an available genome, one option for analysis is *de novo* transcriptome assembly (Fig. 2B). There are many tools available for *de novo* transcriptome assembly and differential expression analysis of these transcriptomes (Davidson, Oshlack, 2014; Grabherr *et al.*, 2011; Haas *et al.*, 2013; Robertson *et al.*, 2010; Schulz *et al.*, 2012) In cases where the reference genome is of low quality, i.e., with misassemblies and large genomic deletions, genes of interest that are absent in the genome assembly can be present in the transcriptome (Park *et al.*, 2014). Another possible approach is mapping assembled transcriptomes to a closely related reference genome, which has been utilized with non-human primates, across the mammalian clade, and the zebra finch and human genomes (Fig. 2C) (Benjamin *et al.*, 2014; Hornett, Wheat, 2012; Vijay *et al.*, 2012). *In silico* mapping to distant reference genomes with up to 15% sequence divergence outperformed mapping to *de novo* transcriptome assemblies, generally recovering more of the transcriptome and reducing the number of mismappings from poorly annotated genes (Vijay *et al.*, 2012). Another study found that mapping to divergent species within 100 million years apart represented more genes than mapping to the transcriptome alone, with similar results to those derived from high quality genomes (Hornett, Wheat, 2012).

Examples of developmental studies using reptilian genome resources

Evolution of genetic pathways regulating somitogenesis in reptiles

There are a number of morphologically divergent features observed in reptiles that are not seen in mammals, particularly in the vertebral column. First, there is an underlying genetic diversity in the regulatory networks that shape vertebral segments that has been revealed through comparative studies adding reptiles in the analysis (Eckalbar *et al.*, 2012; Gomez *et al.*, 2008). Second, there is greater diversity of vertebral segment number and allocation along the body axis (reviewed in Keyte & Smith, 2014; Kusumi *et al.*, 2013; Richardson *et al.*, 1998). Unlike mammals, which are generally constrained to having only seven cervical vertebrae, reptiles display a great diversity of vertebral segment number expansions.

Among tetrapods, there are differences in vertebral morphology and development between the amniotes and amphibians. Since many amphibians have both aquatic and terrestrial life stages, there is development of both a larval spine as seen in tadpoles and subsequent axial reorganization in metamorphosis to adult morphology (Handrigan, Wassersug, 2007; Trueb, Hanken, 1992). In contrast, amniote tetrapods completely form their vertebrae during embryogenesis (reviewed in Rawls, Fisher, 2010). Since the mouse, chick and *Xenopus* frog are developmental model systems, their vertebral development has been well characterized (Burke *et al.*, 1995; Christ *et al.*, 2000; Gossler, Tam, 2002; Ročková, Roček, 2005; Trueb, Hanken, 1992). Molecular studies of axial development have been reported in different species of squamates (Cohn, Tickle, 1999; Eckalbar *et al.*, 2012; Gomez *et al.*, 2008). The evolution of spinal diversity derives from changes in developmental mechanisms controlling the size of vertebral elements, segment number and distribution (lumbar, sacral, caudal, etc.), and embryonic timing (reviewed in Gomez, Pourquie, 2009).

The formation of axial segments, or somites, is regulated by genetic networks regulated by the Notch, Wnt, and FGF pathways collectively called the 'segmentation clock' (reviewed in Kusumi *et al.*, 2013). Most of what we understand about the segmentation clock has been restricted to studies in four model systems (mouse, chicken, frog, and zebrafish) with the following conserved features (EM, O, 2008; Holley, 2007; Krol *et al.*, 2011; Sparrow, 2008): i) Posterior gradients of FGF8, WNT3a, and hairy/enhancer of split

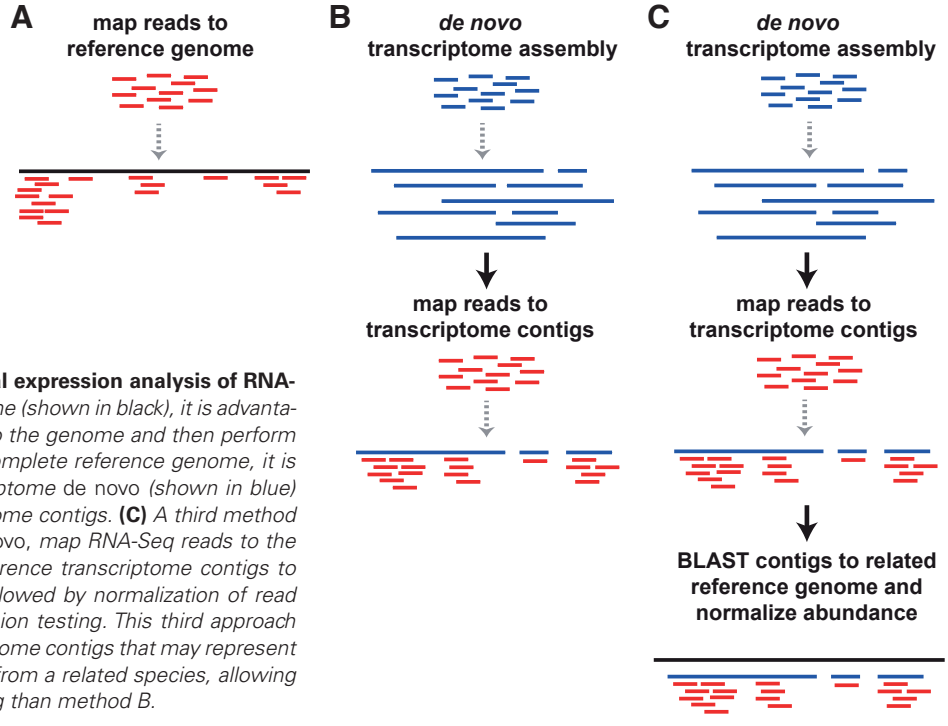

Fig. 2. Read mapping methods for differential expression analysis of RNA-Seq data. (A) *With a complete reference genome (shown in black), it is advantageous to map RNA-Seq reads (shown in red) to the genome and then perform differential expression testing.* **(B)** *Without a complete reference genome, it is possible to first assemble a reference transcriptome* de novo *(shown in blue) and then map RNA-Seq reads to the transcriptome contigs.* **(C)** *A third method is to assemble a reference transcriptome* de novo, *map RNA-Seq reads to the reference transcriptome, and then BLAST reference transcriptome contigs to a closely related complete genome. This is followed by normalization of read abundance and counts and differential expression testing. This third approach makes it possible to combine multiple transcriptome contigs that may represent the same gene and to use robust annotations from a related species, allowing for more accurate differential expression testing than method B.*

(HES and HER) proteins and rostral gradient of retinoic acid in the unsegmented paraxial mesoderm, ii) cyclical expression of genes in the Notch, Wnt, and FGF pathways, iii) the *mesp2* gene that integrates segmentation network gene information at the determination front. In the segmentation clock, information from gradients of gene expression within the presomitic mesoderm (PSM) is integrated with the expression of genes that are cyclically transcribed in that tissue. Somite boundaries are determined based on the periodic interaction of the cycling genes and these gradients. These four model organisms of focus for previous studies of somitogenesis do not capture the full diversity of vertebrates. Analysis of somitogenesis in the green anole lizard and the American alligator identified convergence in cycling expression (lunatic fringe in both mouse and chicken, but not in anole or alligator) and conservation of genes expressed in gradients in the presomitic mesoderm in both squamates and anamniotes (*hes6* in green anole, *Xenopus*, and zebrafish) (Eckalbar *et al*., 2012).

Axial identity and boundaries of Hox gene expression are also set during somitogenesis (Alexander *et al*., 2009; Zákány *et al*., 2001). Mutations in the Notch pathway effector *Rbpj* were shown to disrupt the dynamic expression of *Hoxd1* and *Hoxd3*, and, in transgenic mice with dominant negative alleles of *Dll1* that have reduced Notch signaling in the PSM, there are homeotic vertebral transformations and subtle changes of *Hox* gene expression (Cordes *et al*., 2004). In homozygous *Lfng* null mutants and in transgenic animals overexpressing *Lfng*, vertebral identities were altered, numbers of segments in the cervical and thoracic regions were reduced, and expression of *Hoxb6* was shifted rostrally. Altogether, these findings confirm that the segmentation process is coupled to the determination of axial identity through Notch pathway regulation of *Hox* expression.

Snakes are some of the most striking examples of both increased number of vertebral segments combined with loss of limbs (Caldwell, 2003; Gans, 1975; Greer, 1987; Greer, 1991; Lande, 1978). The emergence of a "snake-like" morphology is estimated to have arisen independently at least twenty-five times in the squamates (Brandley *et al*., 2008; Wiens *et al*., 2006). Molecular studies of the corn snake identified that generation of over 300 vertebral segments was associated with both increased rate of the segmentation clock rate together with increased formation of presomitic mesoderm in the tailbud (Gomez *et al*., 2008). There was also an expansion in expression of thoracic Hox genes in the python (Cohn & Tickle, 1999). With the whole genome sequencing of additional squamates, we will better understand whether common or divergent genetic regulatory changes are driving the repeated evolution of "snake-like" morphology.

Regeneration in lizards

Regeneration of appendages occurs throughout vertebrates, though the extent of regeneration varies throughout taxa (Bely, Nyberg, 2010). Amphibians and teleost fish are spectacular examples of limb and tail regeneration (Stocum, Cameron, 2011). Many lizards are capable of tail regeneration following tail amputation and/or autotomy, and tail regeneration in alligators has been reported in the field (Han *et al*., 2005). Birds and mammals have limited regenerative capacity in comparison, though some neonatal and juvenile mammals can regenerate digit tips, and African spiny mice can autotomize and regenerate skin (Han *et al*., 2008). As amniotes, lizards are the most closely related organisms to mammals that can regenerate whole structures, and the green anole has a reference genome and robust annotation (Alföldi *et al*., 2011; Eckalbar *et al*., 2013), allowing for transcriptome-wide studies of molecular pathways and mechanisms involved in lizard tail regeneration.

Though the regenerating tail has a different structure than the original tail, it is an impressive example of regeneration of cartilage, *de novo* muscle groups, skin, vasculature, and neural ependymal cells (Fisher *et al*., 2012; Gilbert *et al*., 2013; Hutchins *et al*., 2014; McClean & Vickaryous, 2011; Ritzman *et al*., 2012). While blastema formation is fairly well characterized during limb and fin regeneration in amphibians and teleost fish, lizards follow a different mechanism of regeneration. Blastema formation is traditionally characterized by dedifferentiation of tissue, proliferating cells focused at the tip of the regenerating appendage, and the absence of a vascular bed (Iten & Bryant, 1973; Mescher, 1996; Peadon & Singer, 1966; Singer, 1974; Smith & Wolpert, 1975). However, there is no evidence of dedifferentiation in the lizard (Cox, 1969; Fisher *et al*., 2012; Hughes & New, 1959; Hutchins *et al*., 2014; Simpson, 1965). Additionally, in the leopard gecko (*Eublepharis macularius*) and green anole (*A. carolinensis*), proliferating cells are present throughout the regenerating tail, and the distal tip is highly vascular (Hutchins *et al*., 2014; McClean, Vickaryous, 2011).

Though there is a lack of evidence for blastema formation in regenerative squamates, studies of the molecular basis of tail regeneration have shown many shared pathways with other vertebrates (Hutchins *et al.,* 2014). There are hundreds of genes that are differentially expressed along the proximal-distal axis of the regenerating tail, including those related to wound healing, musculoskeletal development, hormonal response, embryonic morphogenesis, and the Wnt and MAPK/FGF signaling pathways. The Wnt pathway in particular has been identified as a key regulator of regeneration in the salamander limb blastema (Knapp *et al*., 2013; Wu *et al*., 2013) and mouse digit tip (Takeo *et al*., 2013). It is possible that all vertebrates have inherited the innate genetic and regulatory repository associated with regeneration. What is unclear is why some lineages, such as mammals, have lost the ability to regenerate in the adult stage despite conserving the genes involved in regrowth. Unlike anamniote models zebrafish or salamander, lizards can provide information on amniote-specific pathways and patterns necessary for regeneration.

Carapace and plastron formation and tooth loss in turtles

The shell is a novel phenotype that unites all turtles, and comprises of a set of highly derived morphologies which combine to create a bony shield on both the dorsal (known as the carapace) and ventral sides (known as the plastron) of the animal. While fossil turtles are well known due to the fact that their hard and bony shells fossilize quite readily, the very early and rapid appearance of a complete shell in turtle evolutionary history has contributed to a relative lack of transitional forms in the fossil record. The oldest known turtle, *Odonotochelys*, (Li *et al*., 2008) was found in 220 million year old deposits in China and has a complete plastron and an under-developed carapace. This pattern matches the emergence of the turtle shell during embryonic development, which diverges significantly from the more conserved ancestral amniote condition (Gilbert, 2001) The painted turtle genome revealed significant gene family expansions in beta-keratins which play an important role in the formation of the shell, and mRNAs extracted from *Pseudemys*

nelsoni shell precursor cells revealed independent patterns of beta-keratin involvement in turtle shells and bird feathers (Shaffer *et al.*, 2013). Cross-species gene expression profiling between chicken and softshell turtle embryos suggest a conserved vertebrate phylotypic period, followed by significant turtle-specific repatterning of 233 genes whose gene ontology categories include ossification and extracellular matrix regulation, as well as crucial roles of 212 microRNAs and a co-option of the Wnt signaling pathway in the development of the carapacial ridge (Wang *et al.*, 2013). Another key trait of turtles that differs from other reptiles is tooth loss, which has been associated with extensive generation of pseudogenes, including degradation of tooth-specific genes such as enamelin (*ENAM*), which contains multiple stop codons and non-conserved sequence (Shaffer *et al.*, 2013). The availability of genomic resources for turtles will continue to shed light on the development of the characteristic traits of this enigmatic group.

Development and evolution of the archosaurian heart

The septation of the heart tube to form a four-chambered heart arose independently in mammals and in archosaurian reptiles. The emergence of this developmental septation process represents a well-known case of evolutionary convergence (Farmer, 1999). While it makes sense that the metabolic demands of flight would lead to cardiac septation in birds, modern crocodilians have a much more ectothermic "reptilian" lifestyle, and the four-chambered heart is likely a vestigial trait that was ancestral to highly active and likely endothermic stem archosaurs. A study of non-crocodilian reptiles (the turtle *T. scripta* and the green anole *A. carolinensis*) that focused on gene expression in developing ventricles showed that turtles and lizards initially form a ventricular chamber which homogeneously expresses the *Tbx5* transcription factor, while in chicken and mouse *Tbx5* expression is restricted to a left ventricle and excluded from the prospective right ventricle (Koshiba-Takeuchi *et al.*, 2009). Transgenic ectopic expression of *Tbx5* in the prospective right ventricular region of mice led to loss of the ventricular septum, and changes in genetic regulation of *Tbx5* are thought to have arisen independently in the avian and mammalian lineages.

How will further reptile genomes advance comparative developmental studies?

Next-generation sequencing technologies promise to increase the number of reptilian genomes – adding to the currently available four lepidosaurs, three turtles, and four crocodilians – to allow the research community to address many unresolved questions using comparative methods. While it has been shown that the mapping of transcripts to a moderately distantly related reference genome could prove useful, highly divergent genes, which can be of great interest, are underrepresented in analyses without an available reference genome. Using only transcriptomes, it is difficult to study cis- regulatory elements, copy number variation, transposable elements, and noncoding RNAs, which can be important regulators of gene expression. The continuing availability of reptile genomes will provide more resources for comparative gene expression studies. For instance, squamates are sorely unrepresented as there are currently four available complete genomes out of >9,400 species. Squamates as a group contain many convergent phenotypes, such as leglessness, and their genomes would be a prime resource for understanding the development of axial and appendicular morphologies. Given the ever-increasing pace of ease and affordability of genome sequencing projects in the next-generation sequencing era, it is likely that the current gaps in phylogenetic sampling across reptiles will begin to get bridged, and a true appreciation for the diversity of forms across amniotes will emerge.

Acknowledgements

The research carried out by the authors and reviewed in this publication was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Number R21 AR064935 and funding from Arizona State University. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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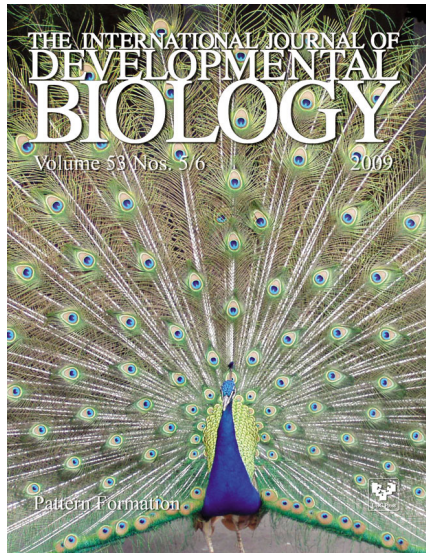

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