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John A. J. A. St John, Edward L. E. L. Braun, Sally R S. R. Isberg, Lee G. L. G. Miles, Amanda Y. A. Y. Chong, et al.. Sequencing three crocodylian genomes to illuminate the evolution of archosaurs and amniotes. *Genome Biology*, 2012, 13, online (1), Non paginé. 10.1186/gb-2012-13-1-415 . hal-01000270

HAL Id: hal-01000270

<https://hal.science/hal-01000270>

Submitted on 29 May 2020

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OPEN LETTER

Sequencing three crocodylian genomes to illuminate the evolution of archosaurs and amniotes

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Abstract

The International Crocodylian Genomes Working Group (ICGGWG) will sequence and assemble the American alligator (*Alligator mississippiensis*), saltwater crocodile (*Crocodylus porosus*) and Indian gharial (*Gavialis gangeticus*) genomes. The status of these projects and our planned analyses are described.

Keywords Genomics, evolution, Crocodylia, Archosauria, amniote

The importance of reptilian genomics

The study of reptilian genomes is essential if we are to understand the patterns of genomic evolution across amniotes (mammals, birds and non-avian reptiles). Non-avian reptiles differ from mammals and birds in several ways: they have diverse sex-determining systems, are exothermic ('cold blooded') and have extreme physiology. Non-avian reptiles are divided into four extant orders: Crocodylia (crocodiles and alligators; approximately 25 species), Sphenodontia (tuatara; two species), Squamata (lizards and snakes; approximately 7,900 species) and Testudines (turtles; approximately 300 species). The clade's most recent common ancestor is thought to have lived around 275 million years ago (Mya) [1], and birds (class

Aves) are nested within reptiles (class Reptilia) (Figure 1). Although they are more diverse than birds and mammals, non-avian reptiles have not been a major focus of genome sequencing efforts [2,3]. The green anole (*Anolis carolinensis*) is the only non-avian reptilian genome sequence published to date [4]. There are, however, ongoing initiatives to sequence the genomes of the painted turtle (*Chrysemys picta*; see NHGRI Genome Sequencing Proposals [5], the garter snake (*Thamnophis sirtalis* [6]), the king cobra (*Ophiophagus hannah*; M.K. Richardson, personal communication and the Burmese python (*Python molurus bivittatus* [7]). Although these projects will provide considerable insight into the evolution of both reptilian and amniote genomes, they only begin to address the diversity represented within reptiles, and do not include any crocodylians.

Order Crocodylia is a key group within Reptilia and genome drafts from crocodylians would provide insights into ancestral reptilian and amniote genomes. These genome assemblies will also enable more detailed inferences on the evolution of three additional lineages of substantial interest to vertebrate biologists: dinosaurs, pterosaurs and birds. Crocodylians and birds are the only extant members of Archosauria (a clade that also includes dinosaurs and pterosaurs along with several extinct lineages) [8]. Among archosaurs, only the genomes of chicken (*Gallus gallus* [9]), turkey (*Meleagris gallopavo* [10]) and zebra finch (*Taeniopygia guttata* [11]) have been sequenced, although several additional avian genomes, such as the Mallard duck (*Anas platyrhynchos* [12], budgerigar (*Melopsittacus undulatus*, a type of parrot) and a set of other avian taxa [13] are currently underway [14]. Crocodylians are the best extant outgroup for comparative analysis of avian genomes, and, as such,

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would substantially enhance analyses of the large set of bird genomes that are expected to be available shortly. Avian and crocodylian genomes provide the best hope for elucidating the gene and genomic properties of dinosaurs and other extinct archosaurs, about which we have learned surprising amounts (for example, genome size and limited protein sequences) considering we have no access to the DNA of these organisms [15-19]. In the broadest sense, Crocodylia represent an important vertebrate clade, and their genomes hold information that will illuminate the underlying relationships among all amniotes. In addition, crocodylians present several interesting biological questions that can be approached from a genomic perspective, many of these will be discussed below.

Background on crocodylians and project justification

The order Crocodylia, which typically refers to the clade that includes the extant crocodylians [20], is an ecologically successful group of reptiles that originated in the mid- to upper-Cretaceous period (approximately 100 Mya) [21,22]. Crocodylians are apex predators in the marine and freshwater habitats where they reside. They play a major role in warm-water ecosystems throughout the world. Extant crocodylians are members of a larger group, termed the Crocodylomorpha, that appeared in the fossil record by the upper Triassic (about 200-250 Mya) [8,1], a date coincident with molecular estimates of the avian-crocodylian divergence [2,22,23]. Crocodylia is divided into three families with extant members, Alligatoridae (alligators and caimans), Crocodylidae (crocodiles) and Gavialidae (gharials) [21,23]; the Gavialidae are traditionally thought to be the outgroup of a clade comprising Alligatoridae and Crocodylidae [21]. However, recent phylogenetic analyses of both molecular data [22,24] and combined molecular and morphological data [25] support a closer relationship between Crocodylidae and Gavialidae (Figure 1).

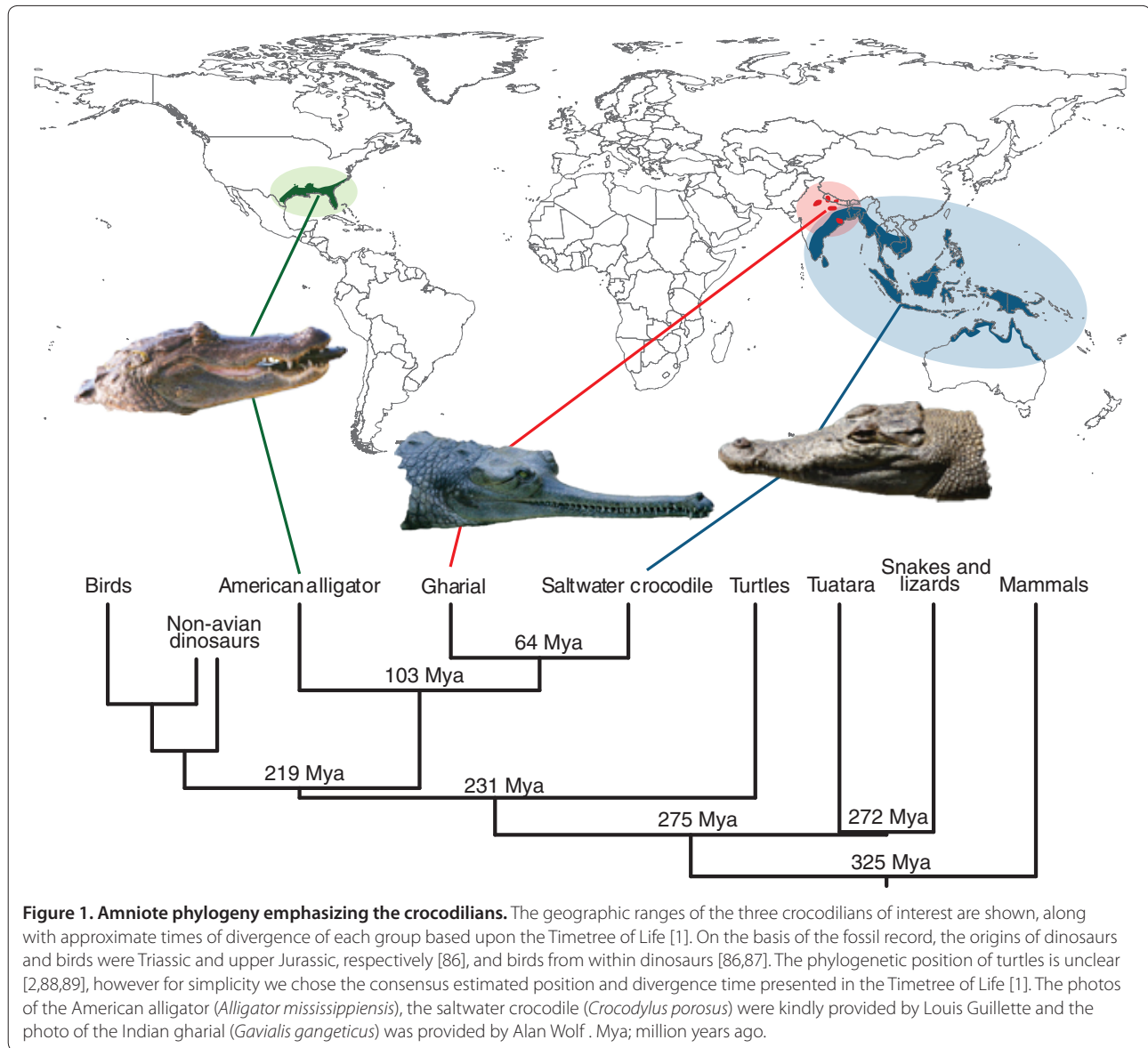
Crocodylians have been a part of the human narrative for centuries, appearing in modern popular culture (for example, the wildlife documentary series *The Crocodile Hunter*), scientific documentaries, as ancient mummies and in cave paintings. They are prized for their hides and meat, and some species, such as the American alligator, the Nile crocodile (*Crocodylus niloticus*) and the saltwater crocodile, are ranched (that is their eggs are brought in from the wild) and/or farmed (in which captive breeding stock produce the eggs). Globally, crocodylians are a source of trade worth more than \$US500 million [26]. However, crocodylians likely have their most profound economic impact as tourist attractions [27,28]. Thoughtful ecotourism could be the best hope for saving endangered crocodylians, such as the critically endangered gharial, from extinction and their habitats from destruction.

Given their popularity, their status as the sister group of dinosaurs, and their inherent public fascination, efforts focused on crocodylian genomics are ideally suited for education and outreach focused on evolution and comparative genomics. Indeed, the preliminary data from our efforts has been used in a pilot genomics course at the University of Florida that integrates with undergraduate research. The consortium plans to make material for genomics pedagogy and public outreach available in parallel with the release of the genome assemblies.

In addition to their ecological, sociological and economic significance, crocodylians have genomes that will be useful sources of data for biological and biomedical research. Alligator serum has been shown to contain broad spectrum antibiotic peptides [29-32]. The American alligator has been used extensively as a model for examining the environmental impact of various contaminants, including endocrine disrupting xenobiotics [33-36]. Crocodylians represent important research organisms for diverse fields that include evolution and phylogenetics [25,37-39], functional morphology [37,40], osmoregulation [37], sex determination [41-45], hybridization [46-48] and population genetics [49-51]. To provide the genomic resources necessary to expand our understanding of these fascinating organisms, the ICGWG is obtaining and assembling genome sequences for the American alligator, saltwater crocodile, and gharial, one representative from each of the extant crocodylian families. For further information about the project and preliminary assemblies, see Ref. [52].

Properties of crocodylian genomes and available genomic resources

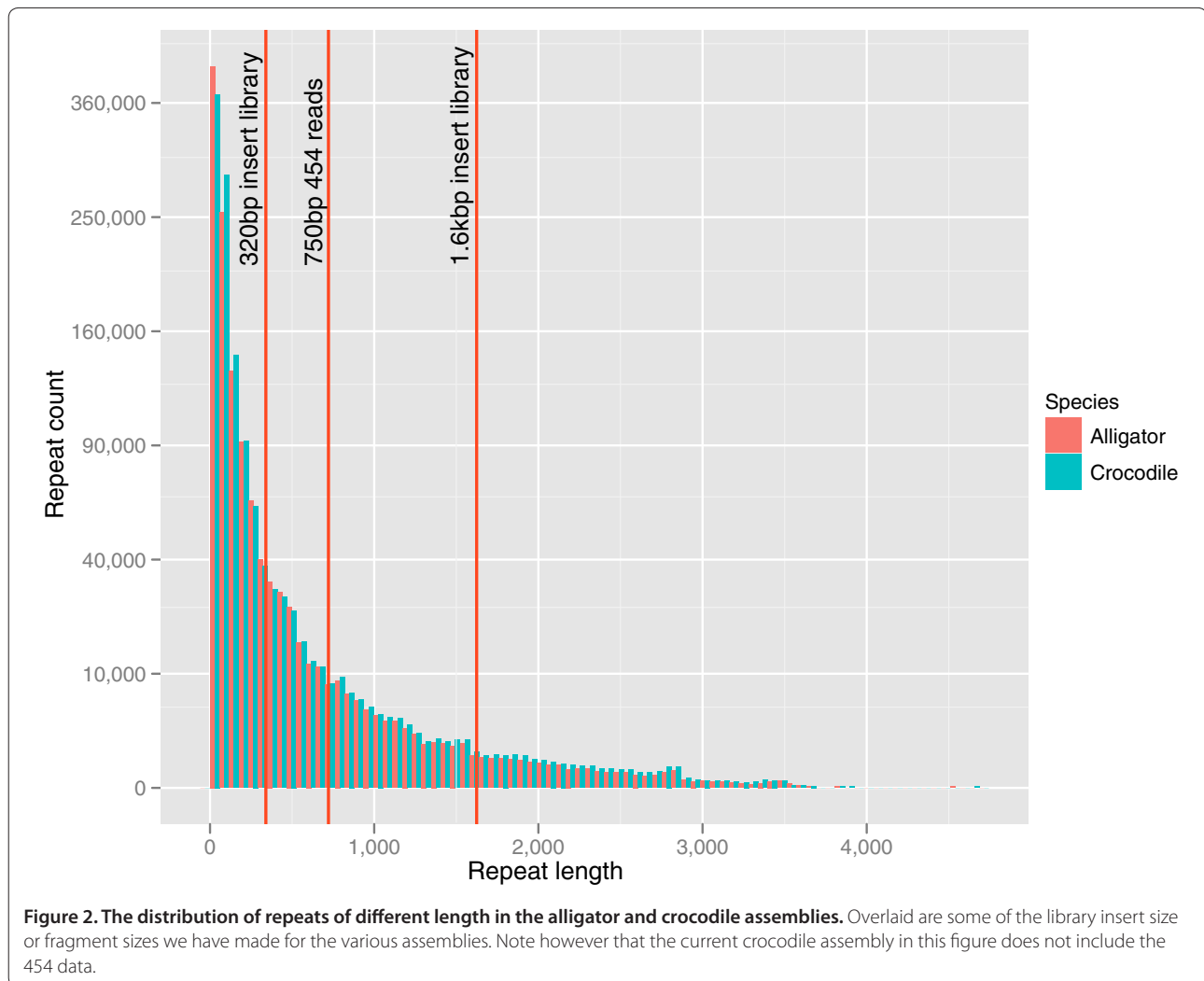
Short of whole genome sequencing, much work has been done on crocodylian genomes, especially the American alligator and Australian saltwater crocodile. The genome of the American alligator is approximately 2.5 gigabases [53] comprising 16 pairs of chromosomes [54,55]. The genome size of the saltwater crocodile is around 2.78 gigabases [56] with 17 pairs of chromosomes [54,57]. The genome size of the gharial is currently unknown, although it is likely to be approximately 2-3 gigabases, given the genome sizes of other crocodylians. Like the American alligator, the gharial has 16 chromosomes [54]. Unlike organisms with genetic sex-determination systems, crocodylians are not thought to have sex chromosomes [54]. Instead sex is determined by incubation temperature of the egg [42]. Although microchromosomes are common among other reptiles (including birds), and there is striking variation in chromosome sizes within crocodylians, the smallest crocodylian chromosomes are not generally regarded as small enough to be classified as microchromosomes [54,58,57,55].



As in birds, the most common transposable elements (TEs) in crocodylian genomes are Long Interspersed Elements (LINEs) of the chicken repeat 1 (CR1) family [59]. Earlier studies indicated that the majority of CR1 LINEs in crocodylians are fairly short (typically <2kbp [59]). Indeed, our efforts to identify novel repeats in preliminary saltwater crocodile and American alligator genome assemblies show that the most abundant repeats in the current assemblies are less than 1kbp (Figure 2). The observation that this relatively well-characterized and short class of TE insertions is the predominant family of repeats in crocodylians suggest that assembling the genomes of these organisms will be a manageable project, compared with a typical repeat-rich mammalian

genome that contains a greater proportion of longer repetitive elements.

Libraries of bacterial artificial chromosomes (BACs) are available for all three species of interest and these will be used for each genome project. The American alligator BAC library currently has about 10X clone coverage [60], the saltwater crocodile library has approximately 3.7X clone coverage [56] and the gharial library has about 5.7X clone coverage, assuming it is a 2.7 gigabase genome (X. Shan, unpublished data). Several large-scale nucleotide datasets have been collected for the American alligator, including 21 assembled BAC sequences completed through the NISC Comparative Sequencing Initiative [61], and 3,276 Sanger BAC-end reads [59]. A



linkage map based on microsatellite loci [62] for the saltwater crocodile is also available. Additionally some saltwater crocodile microsatellite loci have been mapped by fluorescence *in situ* hybridization (FISH) to physical chromosomes using fosmids and BACs ([58] and P. Dalzell unpublished data), which will facilitate anchoring portions of the genome assembly to chromosomes.

In addition to genomic sequences and mapping information, both Sanger and 454 transcriptome data for the crocodile and alligator are available [63,64]. Transcriptome data will be further augmented by a diversity of tissue-specific cDNA libraries from multiple species that will be sequenced using Illumina RNA-seq to assist gene annotations. The cDNA sequences will also enable further scaffold ordering and orientation for transcripts that are split between multiple genomic fragments [65]. We will use these legacy and new data to further improve the initial *de novo* assemblies. To view the preliminary assemblies, see Ref. [52].

Sequencing strategy for the three crocodylian genomes

Owing to the availability of diverse legacy data, we are pursuing different strategies for the sequencing and assembly of each genome, as described below.

For the American alligator genome, we are following the Allpaths-LG recommended pipeline [66] of a combination of high coverage pairs of overlapping reads with a second, moderate coverage, longer insert mate-pair library. This pipeline has yielded good results with a variety of assemblies including *de novo* reassemblies of mouse and human [66], and was successfully employed in an independently evaluated genome assembly contest [67]. We have combined approximately 50x coverage from an overlapping, Illumina, short-insert library with about 20x coverage from an Illumina 2kbp mate-pair library. To investigate genetic variation and increase coverage, we will combine these reads with a set of short, non-overlapping 2x100 bp Illumina reads at approximately 50x

coverage. In addition to providing deeper coverage, these data will also provide information about genetic variation in American alligators due to single nucleotide polymorphism differences between the diploid chromosomes of an individual. We will further scaffold the assembly using low coverage BAC-end sequences, and we will carry out FISH mapping to assign scaffolds to chromosomes.

To sequence the saltwater crocodile genome, we are combining high coverage Illumina short insert sequencing with low coverage 454 libraries in a hybrid approach, similar to that used for the turkey genome [10]. We currently have about 80x coverage from a non-overlapping, short-insert library and an additional 40x from an overlapping short-insert library. We also plan to generate about 20x coverage from an Illumina 2kbp mate-pair library. To supplement the Illumina data, we have generated 1x coverage of unpaired 454 reads (about 700bp in length), and plan to generate an additional 2x coverage from 3kbp and 6kbp paired 454 reads. We will also end-sequence the crocodile BAC library using a method similar to the fosmid-based ShARC method described by Gnerre *et al.* [66]. Some of these BACs are known to contain microsatellite DNA markers used in the crocodile linkage map [62] and others have already been FISH mapped to chromosomes in the crocodile [58]. We will integrate this information for scaffolding and assigning scaffolds to chromosomes. As with the American alligator genome, we are also generating transcriptome data for the saltwater crocodile for both annotation and scaffolding purposes. We will also use the 454 brain transcriptome data that exists for the American alligator [64] and the Nile crocodile [68] in our analyses. We will use these EST and RNA-seq data, along with the other resources described above, to further order and orient scaffolds within the assembly.

Finally, we will assemble the gharial genome using a hybrid approach similar to that used for the saltwater crocodile. To do this, we have generated 40x coverage from an overlapping short-insert library. This will be combined with sequences from 400 bp and 700 bp paired-end Illumina libraries sequenced to give approximately 30x coverage, as well as 2-3x genome coverage consisting of 454 shotgun reads and 3kbp and 6kbp paired-end 454 libraries with FLX+ reads. Finally we will generate approximately 20x coverage from an Illumina 2kbp mate-pair library. The gharial is a critically endangered species, making it nearly impossible to collect a wide variety of tissues for transcriptome data. Nonetheless, we have collected blood, which will be used to generate Illumina RNA-seq data. As with the American alligator and saltwater crocodile, we will use *de novo* assembled transcripts to improve the assembly.

Project timeline and goals

The first phase of our sequencing effort, in which we generate high coverage short insert and overlapping libraries, has been completed for American alligator and saltwater crocodile and is ongoing for the Indian gharial. The data generated for alligator and crocodile were used to generate early draft assemblies for those genomes. The second phase will involve generating longer distance mate-pair libraries and BAC-end sequences to improve the assemblies. We plan to have the data gathered for this phase by mid-March 2012. The third and final phase will involve FISH mapping the BACs to assign scaffolds to chromosomes. When all three phases are completed the assemblies should be as contiguous as possible, given the combination of high coverage short distance information generated in phase one with lower coverage long distance information generated in phase two. The third phase is not critical for the most pressing questions involving crocodylian genomics; individual genes and their regulatory regions will be of primary interest, as opposed to the long-range linkage required for identifying selective sweeps. Thus we will proceed with this third phase in parallel with our other comparative genomic analyses. Once the three genomes are assembled, we will perform comparative genomic analyses both within Order Crocodylia, and among crocodylians and other members of Reptilia.

The completion of each of these phases will be publicly communicated via the website, and links to the data and assemblies will be available to researchers with restrictions as detailed below. We anticipate data collection and initial analyses to be complete by June 2012, and we plan to submit the genome paper within one year of finalizing these initial analyses. The Toronto Statement [69] suggests that there be a one-year period of initial analyses and publication, after which the broader community would be free to use this data in an unrestricted manner. Precise dates at which we complete data collection and initial analysis, and thus the beginning of the embargo period on the genome data, will be promptly posted on the website [52].

Status of the current preliminary genome assemblies

Preliminary assemblies for alligator and crocodile are available. The assembly for alligator additionally uses information from a 120x physical coverage, Illumina 1.5kbp mate-pair library. The current crocodile assembly was generated with 80x coverage from a 380bp paired-end Illumina library. The statistics for the length and contiguity of these two assemblies are shown in Table 1. These assembly statistics are on par with other early stage *de novo* assemblies using short read data [7,70].

To obtain early estimates of potential TE content, we analyzed the current assemblies using RepeatMasker and

Table 1. Overview of the current draft assemblies^a

Genome	Estimated Length (Gbp)	Assembly Length (Gbp)	Estimated % Coverage	Contig N50 (Kbp)	Contig N90 (Kbp)	Scaffold N50 (Kbp)	Scaffold N90 (Kbp)
American alligator	2.5	2.17	86.8	28.0	6.9	106.2	22.5
Seawater crocodile	2.78	2.14	77.0	13.3	3.0	28.2	6.6
Indian gharial	2.5	N/A ^b	N/A ^b	N/A ^b	N/A ^b	N/A ^b	N/A ^b

^aStatistics of the current draft assemblies assuming the conversion between C-value and bp is 0.987×10^9 bp/pg [90]. For this table, we calculated N50 in terms the size of our assembly rather than the estimated genome size. ^bN/A: not available as the genome sequencing and assembly is in progress.

a custom repeat library. The library consisted of all vertebrate TEs identified in RepBase [71] and a set of potential TEs identified by applying RepeatScout [72] to both raw 454 data and to the current assemblies (D. Ray, unpublished data). Consistent with earlier studies [59,73,74], much of the repetitive content of the genome comprises non-long terminal repeat (non-LTR) retrotransposons from the CR1 family (Figure 3). There is also high content of Chompy-like miniature inverted-repeat transposable elements (MITEs) [75], Penelope retrotransposons, ancient short interspersed repetitive elements (SINEs), and satellite/low complexity regions. Overall, 23.44% of the alligator and 27.22% of the crocodile genome assemblies are annotated as repetitive compared with 50.63% seen in humans. Thus, this preliminary analysis provides further evidence that these reptilian genomes might be easier to assemble than typical mammalian genomes due to their lower repeat content.

We also examined GC content across the assemblies (Figure 4). Alligators and crocodiles appear to have a higher mean GC content than many other vertebrates. Additionally their large standard deviation in GC content across contigs is similar to that of birds and mammals, suggesting that their base composition is heterogeneous and likely contains GC-rich isochores. This is unlike the situation in the lizard (*Anolis*) and frog (*Xenopus*), which lack strong isochores based upon analyses of genomic data [76], or the turtle *Trachemys scripta*, which appears to lack strong isochores based upon analyses of expressed genes [77]. However, these results are consistent with previous analyses of ESTs that suggested the existence of GC-rich isochores in the alligator genome [62,77]. Thus, these crocodilian genome data extend the results of the previous analyses and confirm the genome-wide nature of GC-content heterogeneity in crocodilian. We expect improved crocodilian genome assemblies to further illuminate the details of isochore structure in reptiles.

Quality control of intermediate assemblies and raw data

For the alligator genome, we have collected nearly 1.8 billion pairs of Illumina reads from embryos at different developmental stages that were incubated at 'male producing' (33.5°C) and 'female producing' (30°C)

temperatures. From these data, we produced a set of rigorously filtered transcript sequences that we will use to assess the completeness and contiguity of the alligator assembly. These transcripts were assembled using the OASES [78] module of velvet [79] as follows. The initial assembly of the RNA-seq paired-end reads produced 749,838 fragments. We identified the longest open reading frames from each and translated them into putative proteins. We then compared these with the set of known protein sequences in the Swiss-Prot database [80], removing proteins that were more than 10% different in length from the full length Swiss-Prot hit, this removed all but 16,972 putative transcripts. We then focused on the CDS sequence of these genes and removed sequences with less than 5x RNA-seq coverage in any 30-bp window of the sequence. This procedure yielded 2,570 high-confidence alligator CDS sequences. We used these sequences to assess the quality and completeness of the current alligator assembly with results shown in Figure 5. Overall, more than 95% of these filtered CDS sequences were full length on a single scaffold. The improvement garnered by subsequent assemblies will be assessed using these data in the same manner. We will assess the quality and completeness of crocodile and gharial genomes in a similar manner.

Because we do not yet have a set of assembled transcripts for the crocodile genome, we instead used a comparative genomics approach for quality assessment on our early assemblies. For example, we generated two pre-release draft saltwater crocodile assemblies, the second of which (here called Crocodile B) had a slightly lower N50 but a greater overall length and slightly greater mean contig size relative to the first version (here called Crocodile A). Because these statistics conflicted, we aligned the two competing versions of the saltwater crocodile genome to the chicken reference genome (UCSC galGal3) using the UCSC multiz genome alignment pipeline [81]. We then analyzed regions of the multi-way alignment that overlapped chicken genes in the n-scan gene track. With these gene alignments we compared the total number of genes that could be aligned across the two assemblies and the overall level of gene fragmentation for the genes that aligned between the two assemblies (Figure 6). Based on this analysis, we

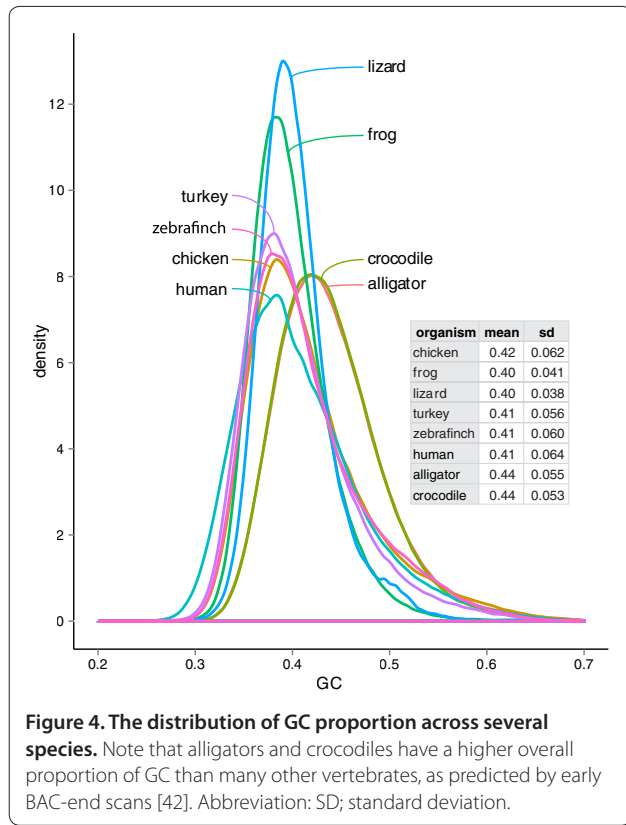


Figure 4. The distribution of GC proportion across several species. Note that alligators and crocodiles have a higher overall proportion of GC than many other vertebrates, as predicted by early BAC-end scans [42]. Abbreviation: SD; standard deviation.

combine the output. We will also partner with groups sequencing additional avian genomes and update the crocodile annotations as needed. Gene finders will initially be trained using the chicken genome and the results from the pipelines will be compared to identify accuracy at both the gene and exon level. Genes will be assigned standardized gene nomenclature based on chicken gene names where there is an unambiguous 1:1 functional ortholog, or a gene identifier in cases where this is not possible. We will also provide preliminary functional annotation for proteins and transcripts using standard Gene Ontology Consortium methods, including functional analysis of motifs and domains and manual curation of orthologs. The ICGWG will perform these analyses to complement and extend those performed by NCBI and Ensembl once the draft genomes are submitted to those organizations.

One major focus will be the large-scale structure of crocodilian genomes, focusing on the degree of syntenic conservation at different scales within these genomes. Karyotype analysis suggests a remarkable conservation of synteny among crocodilians, with the alligator and crocodile having undergone fewer than five chromosomal rearrangements visible at the microscopic level [54] despite 80 million years of evolutionary divergence. However, the level of syntenic conservation at small

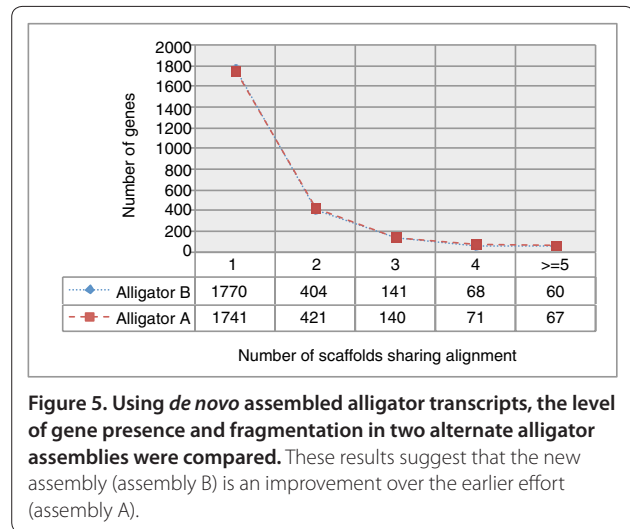


Figure 5. Using *de novo* assembled alligator transcripts, the level of gene presence and fragmentation in two alternate alligator assemblies were compared. These results suggest that the new assembly (assembly B) is an improvement over the earlier effort (assembly A).

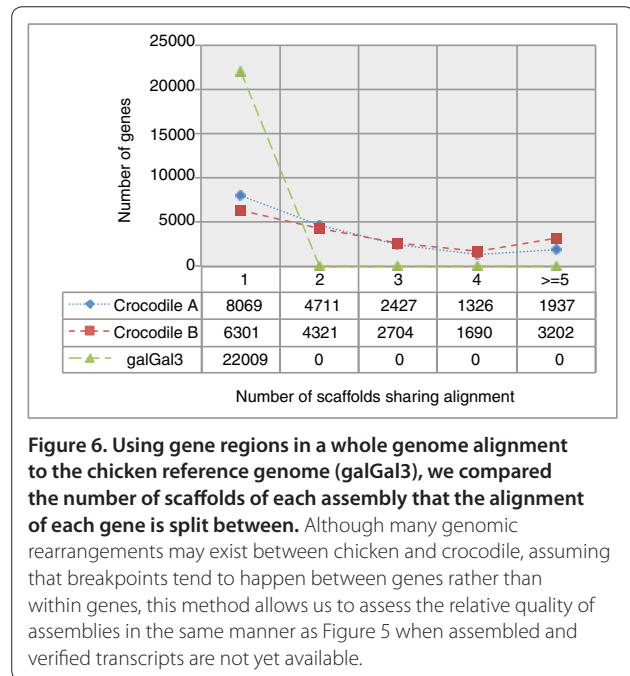


Figure 6. Using gene regions in a whole genome alignment to the chicken reference genome (galGal3), we compared the number of scaffolds of each assembly that the alignment of each gene is split between. Although many genomic rearrangements may exist between chicken and crocodile, assuming that breakpoints tend to happen between genes rather than within genes, this method allows us to assess the relative quality of assemblies in the same manner as Figure 5 when assembled and verified transcripts are not yet available.

scales within these genomes remains unclear, and we expect our genome assemblies to illuminate this topic. Microchromosomes are absent in crocodilians [54,55,59] but present in birds, lizards and snakes, tuatara, and turtles [4,84]. This absence in crocodilians almost certainly represents a derived feature of crocodilians. We will examine the fate of these genetic units within crocodilian genomes. Do microchromosomes comprise linked components within the genomes of the only major reptilian clade without microchromosomes?

Recent work showed that the lizard, *Anolis carolinensis*, unlike other amniotes sequenced to date (with the possible exception of turtles [77]), has a homogeneous genome that lacks GC-rich isochores [76,4]. Our

preliminary analyses indicate that crocodylians have a higher GC-content and greater heterogeneity than *Anolis* (Figure 4), but these analyses are less clear regarding the scale of the observed GC-content variation. Do crocodylians have GC-rich isochores that are similar to those in mammals and birds or do the patterns of GC-content heterogeneity appear distinct?

We will also carry out a number of traditional analyses of genome content using the crocodylian genomes, focusing on repeated sequences and gene families. These analyses include the evolution of repeat families and patterns of TE proliferation. We will compare the repeat family content within crocodylian genomes and with other reptiles and amniotes. Additionally, we will conduct analyses of gene family evolution within reptiles and crocodylians to identify specific genes and other functional elements, including the identification of ultra-conserved regions and potential micro RNA sequences, with a special focus on those sequences that could have been gained or lost both within the crocodylians and in comparison to the other relevant lineages that are now available for investigation.

We will use these three crocodylian genomes to infer their ancestral genome. This, combined with existing and soon to be released bird genomes, will enable some inference of the ancestral archosaur genome. Reconstructing the ancestral archosaur genome has obvious implications for expanding our understanding of the genomes of extinct archosaurs, like the non-bird dinosaurs and pterosaurs (Figure 1).

There are also several biological questions specific to crocodylians that we will address by analyzing genomic and RNA-seq data and via experimental techniques. For example, despite having a temperature-dependent sex-determination system seemingly without sex chromosomes, the sexes of crocodylians have been shown to have very different recombination rates [62]. Identification of the genes that are differentially expressed in the male and female crocodylian gonads might provide insight into the perplexing observation.

SNP discovery arising from the genome sequencing is particularly relevant to farm-bred saltwater crocodiles. Large panels of SNP markers will enable more refined linkage maps [62], more precise mapping of quantitative trait loci (QTL) than is currently possible with microsatellite markers [62] and eventually the implementation of genomic selection in crocodile breeding programs.

Eventually members of the ICGWG hope to address additional questions beyond the scope of the initial genome paper. These might be presented in satellite publications. One of these involves the sex determination system of American alligators. Which genes are the initial temperature sensitive regulators that trigger the downstream, largely conserved [85] sex-determination system?

Having the genome sequences available for these three crocodylians will enable a new wave of discoveries about the evolutionary histories of crocodylians, non-avian reptiles and birds, and amniotes generally.

How other groups can join the consortium, or publish independently with our early release data

This project is affiliated with the Genome 10K (G10K) initiative [14]. We invite other G10K affiliates and the broader scientific community to access and make use of the draft assembly and raw read data that we have produced. Any group performing non-genome-scale analyses that are sufficiently independent of the analyses described above are welcome to use these data without restriction. As a matter of courtesy and to avoid duplicated effort, we request that competing genome-scale projects or analyses that overlap with the areas stated above disclose their status to the ICGWG consortium (formal inquiries and requests to join the working group should be made to D.A.R.) and cite this and subsequent papers that provide the data. Versioned assemblies, further project description, and a complete list of current ICGWG members can be accessed on the website dedicated to this project [52].

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

This work was supported by grants to D.A.R. (MCB-1052500, MCB-0841821, DEB-1020865 from the U.S. National Science Foundation) and funds from the Institute for Genomics, Biocomputing and Biotechnology at Mississippi State University. E.L.B., E.W.T., and collaborators at the University of Florida were supported by funds from the U.S. National Science Foundation (DUE-0920151). T.I. received financial support from the National Institute for Basic Biology and Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan. S.R.I., L.G.M., J.G., P.D. and C.M. were supported by Australian Rural Industries Research and Development Corporation grants (RIRDC PRJ-000549, RIRDC PRJ-005355, RIRDC PRJ-002461). M.K.F. received financial support from a U.S. National Science Foundation Biological Informatics Postdoctoral Fellowship (DBI-0905714). R.E.G. is a Searle Scholar and a Sloan Fellow. E.D.J. was supported by the Howard Hughes Medical Institute and the National Institutes of Health. We are grateful to Kent Vliet (University of Florida) and the Alligator Farm (St. Augustine, Florida) for providing access to fresh gharial blood.

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Published: 31 January 2012

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doi:10.1186/gb-2012-13-1-415

Cite this article as: St John JA, *et al.*: Sequencing three crocodylian genomes to illuminate the evolution of archosaurs and amniotes. *Genome Biology* 2012, **13**:415.