Understanding the Evolution of Reptile Chromosomes through Applications of Combined Cytogenetics and Genomics Approaches

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Abstract

Studies of reptile (nonavian reptiles) chromosomes began well over a century ago (1897) with the initial report on the description of sand lizard (*Lacerta agilis*) chromosomes. Since then, chromosome analysis in reptiles has contributed significantly to understanding chromosome evolution in vertebrates. Reptile karyotypes are also unique, as being the only vertebrate group where the majority of the species possess variable numbers of macro- and microchromosomes, which was first reported for iguanids and teiids in 1921. In addition, many reptiles have microchromosomes as sex chromosomes, highlighting their evolutionary significance, yet very little is known about their evolutionary origin and significance in shaping amniote genomes. Advances in genomic technologies in recent years have accelerated our capacity to understand how sequences are arranged within a genome. However, genomic and cytogenetic analyses have been combined for only 3 species to provide a deeper understanding of reptile chromosome evolution and sequence organiza-tion. In this review, we highlight how a combined approach of cytogenetic analysis and sequence analysis in reptiles can help us answer fundamental questions of chromosome evolution in reptiles, including evolution of microchromosomes and sex chromosomes.

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Reptile cytogenetics played a major role in understanding the evolution of vertebrate karyotypes and genomes with a history spanning over a century, starting from the dawn of cytogenetics. The first described reptile chromosomes were from the sand lizard (*Lacerta agilis*), which was reported by Tellyesniczky [1897]. In 1921, Painter first reported the existence of microchromosomes in iguanind and teiid lizards [for more on the historical perspective on reptile cytogenetics, see Matthey, 1949; Peccinini-Seale, 1981; Olmo et al., 2003 and references therein]. As the discipline of reptile cytogenetics grew over the last century, it became clear that reptiles are an exceptional group in which to study chromosome evolution, as they display a high level of diversity in chromosome number and morphology [Olmo, 2008], differ in the absence or presence of microchromosomes, and have

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diversity in sex determination systems and sex chromosomes [Ezaz et al., 2009; Pokorná et al., 2011; Gamble et al., 2015]. Diploid chromosome numbers range from 2n = 20 in a lizard (Cameroon stumptail chameleon, *Rampholeon spectrum*) to 2n = 68 in a freshwater turtle (twist-neck turtle, *Platemys platycephala*) (Table 1). This level of diversity presents an opportunity to determine types and timing of events that led to the karyotypes of extant species. By comparing chromosomes of species from different reptile lineages, we are able to reconstruct the most likely chromosome arrangement in common ancestors at key positions in the reptile phylogeny. Tracing the evolutionary history of reptile chromosomes will provide insight into the evolutionary processes involved in shaping reptile genomes and the role chromosomal rearrangements have played in speciation. This insight into genome evolution is relevant not only for reptiles, but also for other major lineages of vertebrates because of the key position reptiles hold in amniote phylogeny, having shared a common ancestor with mammals.

Much of the interest in tracing chromosome evolution among reptiles is to gain insight into the diversity of modes of sex determination (genetic sex determination [GSD] vs. temperature-dependent sex determination [TSD]) and the sex chromosomes they possess. From standard karyotyping of species with GSD, it is evident that there are both XX/XY and ZZ/ZW sex chromosomes as well as multiple sex chromosome systems, and these can either be macro- or microchromosomes. Some species have significantly differentiated sex chromosomes, allowing them to be easily identified using standard karyotyping, but other species have cryptic sex chromosomes, requiring more sophisticated approaches for them to be discovered [Ezaz et al., 2005; Martinez et al., 2008]. By comparing reptile genomes, we are able to trace the origin of sex chromosomes and gain general insight in sex chromosome evolution [Ezaz et al., 2017].

Despite the large number of species in this group (over 10,600; the Reptile database, http://www.reptile-database.org), the evolution of reptile chromosomes has not been as thoroughly studied as it has been for mammals and birds [reviewed in Deakin and Ezaz, 2014]. However, molecular cytogenetic studies and reptile genome assemblies with sequence anchored to chromosomes have shed some light on the evolutionary history of reptile chromosomes [Deakin et al., 2016]. Here, we explore the insight cytogenetics and genomics have provided into understanding the evolution of reptile chromosomes and importance of combining cytogenetic and genomic approaches.

Table 1. Ranges of diploid chromosome numbers and numbers of macro-/microchromosomes in major groups of reptiles

Order	Diploid chromosome range	Macro-chromosome range	Micro-chromosome range
Testudines	26–68	10–36	0–56
Crocodilia	30–42	30–42	0
Sphenodontia	36	28	8
Squamata	20–62	10–38	0–36

Molecular Cytogenetic Comparisons of Reptile Chromosomes

Determining the extent of conservation, or conversely, the extent of rearrangement, between species is key to being able to trace the evolutionary history of reptile chromosomes. Based on traditional cytogenetic techniques, such as G-banding, the most conserved reptile karyotypes are observed among crocodilians [Cohen and Gans, 1970; Olmo, 2008] and turtles [Olmo, 2008; Olmo and Signorino, 2005; Valenzuela and Adams, 2011], whereas squamate reptiles (snakes and lizards, including legless lizards) have much higher levels of variability in chromosome number and morphology. Molecular cytogenetic techniques provide greater insight into chromosome evolution and, in some instances, provide comparisons across great evolutionary distances. Despite the extent of information that can be gleaned from molecular cytogenetic approaches, such as chromosome painting and gene mapping, these techniques have only been used on a small number of the thousands of reptile species.

Macrochromosome Evolution
Chromosome painting has been used to determine global levels of homology between different reptilian species and the rearrangements which have taken place between species. These experiments, as for birds, have been limited largely to using probes for macrochromosomes as it has been challenging to flow sort the microchromosomes into pools representing a distinct microchromosome [Griffin et al., 1999; Kichigin et al., 2016]. Conservation of the genomic region corresponding to the chicken Z was determined by hybridizing a chicken Z probe to chromosomes from 30 species, representing 17 squamate families as well as a representative crocodile and turtle. In all except one species, the Z was conserved as a single homologous block [Pokorná et al., 2011]. Following the surprising success of hybridizing a probe to chromosomes

Fig. 1. Comparison of chromosome painting and cytogenetic mapping for turtle and crocodile macrochromosomes and reconstruction of the events leading to macrochromome arrangements in crocodiles [Kawai et al., 2007; Uno et al., 2012; Badenhorst et al., 2015]. Macrochromosomes have been color-coded according to their homology to chicken chromosomes (indicated in gray). Only those macrochromosomes for which there is information on homology to chicken chromosomes are shown.
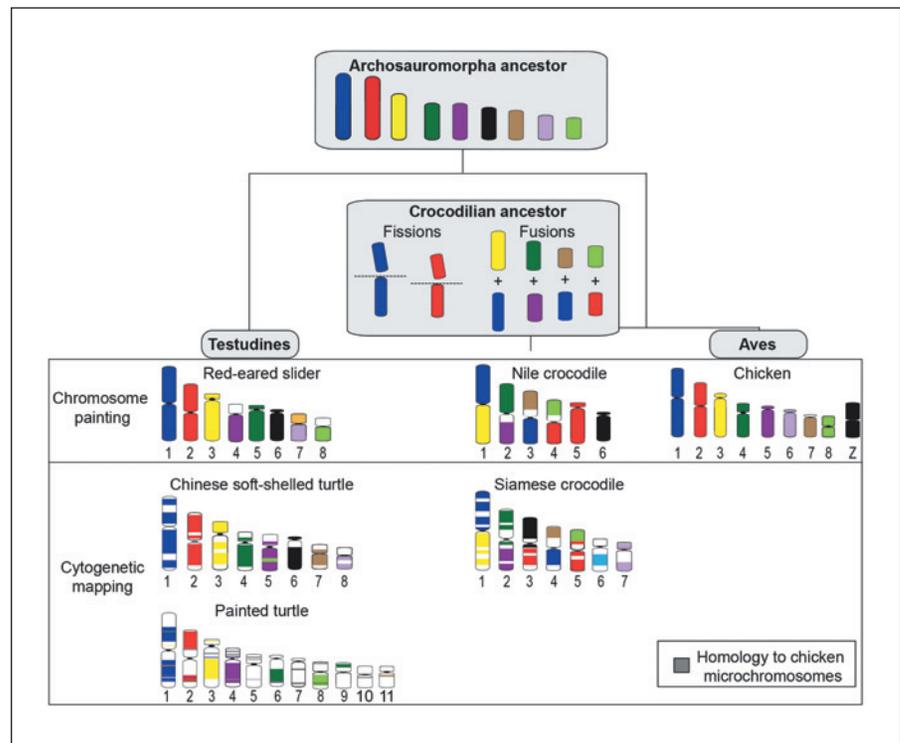
from such distantly related species, probes to other chicken macrochromosomes were attempted. Chicken and red-eared slider (*Trachemys scripta elegans,* 2n = 50) macrochromosomes are remarkably well conserved, considering these species last shared a common ancestor (Archosauromorpha ancestor) over 200 million years ago (Fig. 1) [Kasai et al., 2012]. The same probes hybridized to chromosomes of the Nile crocodile (*Crocodylus niloticus,* 2n = 32) indicating that macrochromosomes 1–6 in this species have been formed by the fission of Archosauromorpha ancestral chromosomes corresponding to chicken chromosomes 1 and 2 and the subsequent fusions of these and other macrochromosomes [Kasai et al., 2012]. Probes for chicken macrochromosomes 3, 5, and 7 have identified homology among 10 species representing 9 families of squamates. In 6 of these species, these 3 chicken probes hybridized to the largest macrochromosome, suggesting a fusion of these took place prior to their divergence from a common ancestor within Squamata [Pokorná et al., 2012].

Cross-species chromosome painting between more closely related species has also been used to determine the level of conservation within families of lizards. For instance, probes generated by flow-sorting chromosomes from the sandfish skink (*Scincus scincus,* 2n = 32) con-

firmed the high level of karyotype conservation among members of the family Scincidae [Giovannotti et al., 2009]. Similarly, paints generated for chromosomes of the Japanese gecko (*Gekko japonicas,* 2n = 38) on 6 other species of gecko, ranging in diploid numbers of 38 to 46, confirmed the typically conserved nature of gecko karyotypes [Trifonov et al., 2011]. Chromosome painting has also been used to identify homology between the mourning gecko (*Lepidodactylus lugubris*) with triploid (3n = 66) parthenogenetic populations, where a chromosome fusion and several fissions from a 2n = 38 ancestor resulted in a 2n = 44 *L. lugubris* karyotype, and parthogenesis has resulted in 3n = 66 karyotypes [Trifonov et al., 2015].

Chromosome painting has provided some insight into macrochromosome evolution among reptiles, but it is unable to detect rearrangements on a finer scale or provide information on the evolution of microchromosomes. Mapping either cDNA or BAC clones to reptile chromosomes using FISH has overcome these limitations to a certain extent by enabling comparative maps to be generated. These maps range from consisting of just 21 markers for the tuatara (*Sphenodon punctatus*) to 356 for the green anole (*Anolis carolinensis*) (Table 2). For 3 species (*A. carolinensis, Pogona vitticeps,* and *Chrysemys picta*),

Table 2. Species with molecular cytogenetic maps

Order	Species	Marker type	Markers, n	Reference
Testudines	*Pelodiscus sinesis*	cDNA	162	Matsuda et al., 2005; Uno et al., 2012
	Chrysemys picta	BAC	61	Badenhorst et al., 2015
Crocodilia	*Crocodylus siamensis*	cDNA	131	Uno et al., 2012
Sphenodontia	*Sphenodon punctatus*	BAC	21	O'Meally et al., 2009
Squamata	*Gekko hokouensis*	cDNA	86	Srikulnath et al., 2015
	Lacerta agilis	cDNA	86	Srikulnath et al., 2014
	Elpaphe quadrivirgata	cDNA	183	Matsuda et al., 2005; Matsubara et al., 2006; Uno et al., 2012
	Varanus salvator macromaculatusi	cDNA	86	Srikulnath et al., 2013
	Varanus exanthematicus	cDNA	17	Srikulnath et al., 2013
	Leiolepis reevesii rubritaneniata	cDNA	54	Srikulnath et al., 2009
	Pogona vitticeps	BAC	174	Young et al., 2013; Deakin et al., 2016
	Anolis carolinensis	BAC	356	Alföldi et al., 2011

these maps have been used to assign genome sequence to chromosomes [Alföldi et al., 2011; Badenhorst et al., 2015; Deakin et al., 2016]. In most cases, different species have been used for chromosome painting studies compared to those used for molecular cytogenetic mapping. This has its advantages and disadvantages. The combination of data from 2 independent sources would validate the results of both approaches. At the same time, using different species increases the information from within an order to provide a deeper understanding of reptile macrochromosome evolution. This is exemplified by comparing molecular cytogenetic data from turtles and crocodiles to chicken to predict the arrangement of macrochromosomes in the Archosauromorpha ancestor, which confirmed the conservation of this ancestral arrangement in turtles and chicken [Uno et al., 2012] as well as the fission and fusion of some of these chromosomes in the ancestral crocodile [Kasai et al., 2012; Uno et al., 2012] (Fig. 1). Additional information from anchoring sequence to chromosomes demonstrates that, despite the remarkable conservation between turtle and chicken macrochromosomes at a broad scale, there has been the incorporation of material corresponding to chicken microchromosomes into *C. picta* macrochromosomes [Badenhorst et al., 2015]. This finding disputes the high level of conservation between chicken and turtle chromosomes previously reported [Badenhorst et al., 2015]. It would, therefore, be interesting to see if this is a common feature amongst turtles or represents rearrangements specific to this species. Similarly, combined molecular cytogenetic data were compared to chicken as an outgroup to predict the macrochromosome arrangement of the squamate, toxicoferan, and iguanian ancestors and the

events leading to those arrangements [Deakin et al., 2016] (Fig. 2).

Microchromosome Evolution

Tracing the evolutionary history of reptile microchromosomes has been more challenging than for macrochromosomes. The presence or absence and number of these tiny chromosomes is responsible for much of the karyotypic variation amongst reptiles, making it important that we decipher their gene content for comparative analysis. Chicken is most often used as a reference genome for reptile comparative genomics, but even for this species, the sequence content of all microchromosomes is yet to be determined [Solinhac et al., 2010]. Cytogenetic maps for reptiles have assigned genes or anchored genome sequence to reptile microchromosomes, but in most cases, this assignment has not distinguished to which microchromosome the marker belongs. For example, the cytogenetic map for *Pelodiscus sinesis* demonstrated that genes on microchromosomes in chicken are located on microchromosomes in the soft-shelled turtle, providing further support for a high level of karyotype conservation between these 2 species [Uno et al., 2012]. However, mapping data for another turtle, *C. picta*, has shown that genomic regions corresponding to chicken microchromosomes are not necessarily on microchromosomes in the painted turtle, with some being located on macrochromosomes [Badenhorst et al., 2015], suggesting that we require much more detailed data to fully understand chromosome evolution in turtles.

Some information on the gene content of lizard microchromosomes is starting to emerge. In combination with the sequencing of the *P. vitticeps* genome, BACs corre-
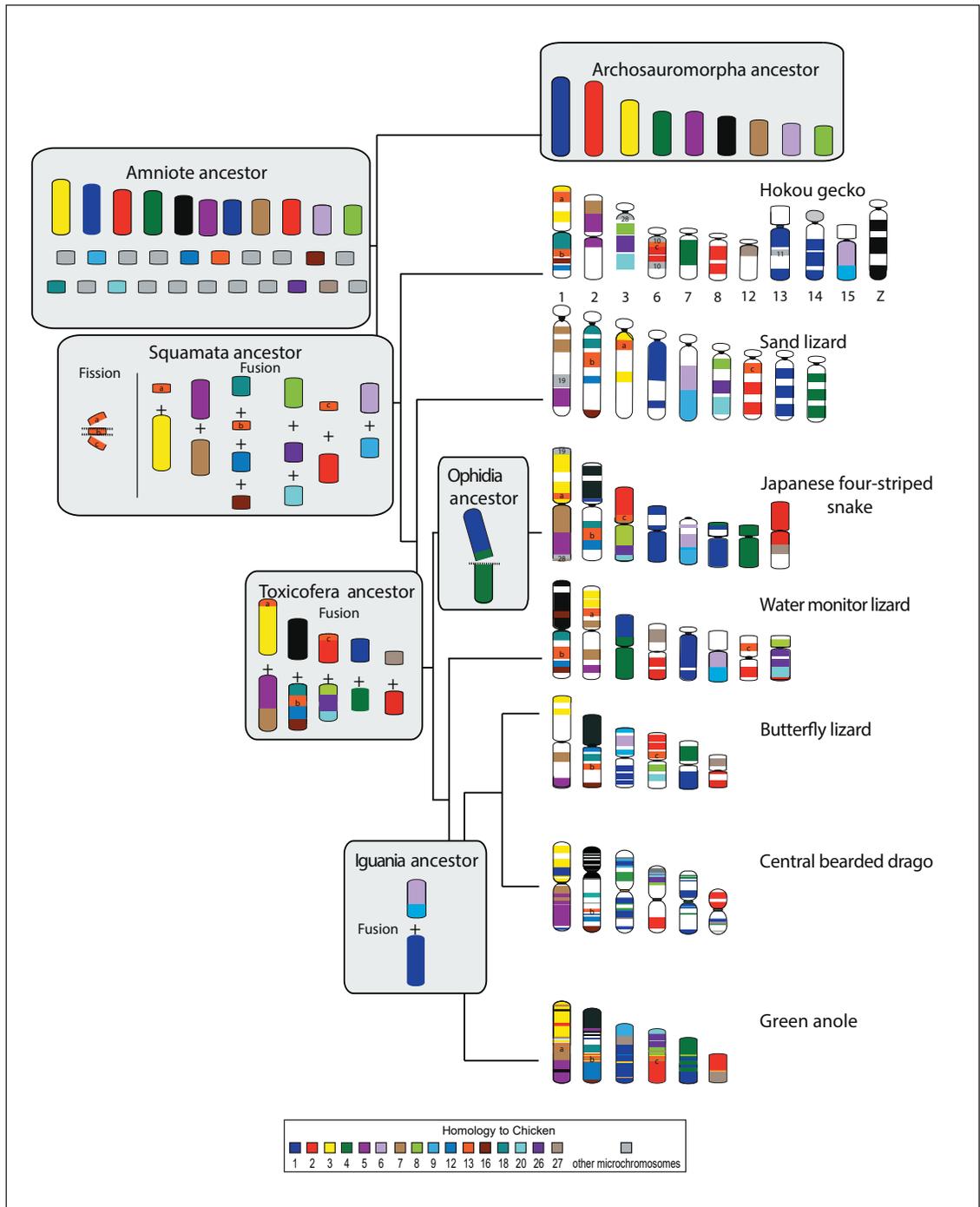
Fig. 2. Reconstruction of the squamate ancestral macrochromosomes from comparisons of combined genome sequencing and cytogenetic mapping data (*P. vitticeps* and *A. carolinensis*) or gene mapping data (all other species). The predicted ancestral karyotypes for the Amniote and Archosauromorpha (crocodiles, dinosaurs, and birds) are based on those previously predicted [Uno et al., 2012]. Only microchromosomes relevant to squamate macrochromosome evolution have been included. Boxes in gray indicate the fissions and/or fusions giving rise to the predicted ancestral karyotypes for the Squamata, Toxicofera, Iguania, and Ophidia. The reconstructed chromosomes have been color-coded for homology to chicken chromosomes.
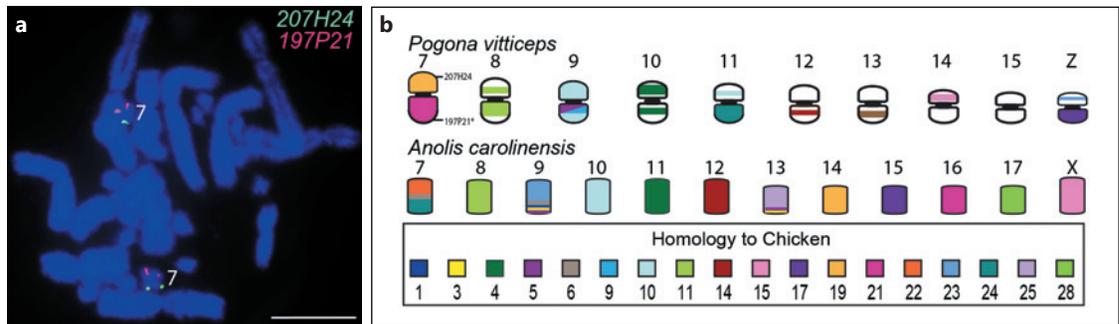
Fig. 3. Deciphering the homology of microchromosomes of 2 sequenced lizard species, *P. vitticeps* and *A. carolinensis*. **a** Assigning a BAC clone corresponding to a *P. vitticeps* genome scaffold using 2-color FISH with anchor BAC 197P21 for microchromosome 7. **b** Homology of *P. vitticeps* and *A. carolinensis* microchromosomes to chicken chromosomes. Content of *A. carolinensis* was determined by sequencing of flow-sorted chromosomes [Kichigin et al., 2016]. The order and orientation of sequence on the chromosomes is unknown. Scale bar, 10 μm.

sponding to the ends of homologous synteny blocks were mapped to dragon chromosomes. BACs mapping to microchromosomes were colocated with an anchor BAC to identify individual microchromosomes (Fig. 3) [Young et al., 2013]. Sequence homology to chicken has been determined for all except the smallest microchromosome (chromosome 15), and most microchromosomes share homology with chicken microchromosomes. The exceptions are dragon chromosome 10, which has some homology to the short arm of chicken chromosome 4, a region predicted to correspond to a microchromosome in the amniote ancestor, and a small region with homology to chicken macrochromosomes 5 and 9 located on dragon microchromosome 9, suggesting that there has been some interchromosomal rearrangement between macro- and microchromosomes [Deakin et al., 2016]. To date, only 8 chicken microchromosomes have been shown to share homology with dragon microchromosomes (Fig. 3). Regions sharing homology with many chicken microchromosmes are located on dragon macrochromosomes, indicating there have been fusions of macro- and microchromosomes at some stage [Deakin et al., 2016] (Fig. 2). Data for other species of lizards are required to determine at which stage during squamate evolution these fusions occurred. In a different approach, Kichigin et al. [2016] used a combination of chromosome painting and sequencing of flow-sorted microchromosomes from 2 anole species (*A. carolinensis* and *A. sagrei*) to determine their homology to chicken. Like those of the dragon, *Anolis* microchromosomes predominantly corresponded to chicken microchromosomes (Fig. 3). Once again, a region sharing homology with chicken chromosome 4 is one of the exceptions.

In species with no (*G. hokouensis*) or a single pair of microchromosomes (*L. agilis*), the microchromosomes have fused mainly with each other to form larger chromosomes, with these fusions potentially arising independently in the Gekkota and Lacertidae lineages [Srikulnath et al., 2014, 2015]. Comprehensive data from more species is required to test this hypothesis.

Moving towards Reptile Chromosomics

From molecular cytogenetic data, we now have a fairly good understanding of the evolutionary history of reptile macrochromosomes and only patchy data for reconstructing the history of microchromosomes, but combined sequencing and cytogenetic approaches are helping to fill in the gaps. Genome sequence also provides a greater level of detail into the extent of rearrangement between species not detectable from sparse cytogenetic mapping data or chromosome painting [Völker et al., 2010; Skinner and Griffin, 2012]. However, the uptake of high-throughput sequencing of reptile genomes has lagged behind that of their avian counterparts, for which over 50 species have already been sequenced [Jarvis et al., 2015]. Only 24 reptile genomes have been published to date, and 9 others have assemblies deposited in the National Center for Biotechnology (NCBI) database (Table 3), but given the reduction in cost and the advances in sequencing technology, it is likely that more will follow in the near future.

Of the 24 published reptile genomes, 3 of the top 6 assemblies with the largest scaffold N50 (the length of the scaffold at which 50% of scaffolds in the assembly are this

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Table 3. Reptiles with sequenced genomes

Species	Common name	Genome size, Gb	Scaffold N50, kb	Reference or GenBank accession
Testudines				
*Chrysemys picta bellii**	Western painted turtle	2.59	6,600	Shaffer et al., 2013; Badenhorst et al., 2015
Chelonia mydas	Green sea turtle	2.24	3,778	Wang et al., 2013
Pelodiscus sinensis	Chinese soft-shelled turtle	2.21	3,331	Wang et al., 2013
Apalone spinifera	Spiny softshell turtle	1.93	2,307	GCA_000385615.1
Terrapene mexicana	Mexican box turtle	2.32	1,387	GCA_002925995.1
Malaclemys terrapin	Diamondback terrapin	2.24	NR	GCA_001728815.2
Crocodilia				
Alligator mississippiensis	American alligator	2.17	10,800	Putnam et al., 2016; Green et al., 2014
Alligator sinensis	Chinese alligator	2.30	2,188	Wan et al., 2013
Crocodylus porosus	Saltwater crocodile	2.12	205	Green et al., 2014
Gavialis gangeticus	Indian gharial	2.88	127	Green et al., 2014
Squamata				
*Anolis carolinensis**	Green anole lizard	1.78	4,033	Alföldi et al., 2011
*Pogona vitticeps**	Central bearded dragon	1.82	2,290	Georges et al., 2015
Deinagkistrodon acutus	Five-pacer viper	1.47	2,122	Yin et al., 2016
Shinisaurus crocodilurus	Chinese crocodile lizard	2.24	1,470	Gao et al., 2017
Ophisaurus gracilis	Asian glass lizard	1.71	1,270	Song et al., 2015
Boa constrictor constrictor	Boa constrictor	1.60	1,135	Bradnam et al., 2013
Gekko japonicus	Japanese gecko	2.55	685	Liu et al., 2015
Eublepharis macularius	Leopard gecko	2.02	664	Xiong et al., 2016
Thamnophis elegans	Garter snake	1.48	647	Vicoso et al., 2013
Thamnophis sirtalis	Common garter snake	1.47	647	GCA_001077635.2
Protobothrops mucrosquamatus	Brown spotted pitviper	1.67	424	GCA_001527695.3
Ophiophagus hannah	King cobra	1.66	226	Vonk et al., 2013
Python molurus bivittatus	Burmese python	1.44	208	Castoe et al., 2013
Vipera berus	Adder	1.53	126	GCA_000800605.1
Anolis auratus	Grass anole	2.02	49	Tollis et al., 2018
Pantherophis guttatus	Corn snake	1.53	36	Ullate-Agote et al., 2014
Anolis frenatus	Bridled anole	2.03	36	Tollis et al., 2018
Crotalus horridus	Timber rattlesnake	1.52	23	GCA_001625485.1
Anolis apletophallus		2.18	10	Tollis et al., 2018
Crotalus mitchellii	Speckled rattlesnake	1.14	5	Gilbert et al., 2014
Sistrurus miliarius	Pygmy rattlesnake	1.31	NR	

NR, not reported. * Sequence cytogenetically assigned to chromosomes.

size or shorter) are those which have included cytogenetic mapping data of BAC clones [Alföldi et al., 2011; Badenhorst et al., 2015; Georges et al., 2015]. The painted turtle, green anole, and dragon lizard genomes have all been useful for reconstructing the evolutionary history of reptile chromosomes. The American alligator genome assembly has been improved since its initial sequencing by using the Chicago method, an approach that provides long-range linkage data from cross-linked DNA, to gen-erate an assembly with an N50 of over 10 Mb [Putnam et al., 2016]. Nevertheless, these longer scaffolds have not been assigned and oriented on alligator chromosomes, which limits the usefulness of this genome for investigating reptile chromosome evolution. Similarly, no attempt has been made to assign the other sequenced reptile genomes to chromosomes, despite cytogenetic mapping data being available for one other species, the soft-shelled turtle [Uno et al., 2012]. For a more complete under-

standing of the extent of genome reshuffling during reptile evolution, we need to take an approach that combines the latest advances in genome sequencing and assembly with cytogenetic data. Such approaches using genome assemblies combined with cytogenetic data have been used to provide detailed genome reconstructions of the genomes of the avian and the eutherian ancestors as well as other key ancestors in these 2 lineages [Romanov et al., 2014; Kim et al., 2017].

The advances in sequencing technology, such as Hi-C (e.g., Dovetail), linked-read sequencing (10X Genomics), and long read (e.g., PacBio and Oxford Nanopore), are bringing chromosome-level assemblies within reach for reptile genomes. When these assemblies are assigned and oriented onto chromosomes, we will be able to perform much more in-depth analyses of reptile chromosome evolution. To date, generating cytogenetic maps for anchoring genomes to chromosomes has been a labor-intensive step, requiring the construction of BAC libraries and library screening or the amplification of cDNA probes. However, recent bioinformatic and cytogenetic advances are making anchoring genomes much more achievable. Universal BAC probes, identified bioinformatically through multigenome alignments to increase the success of cross-species FISH, and the development of high-throughput, cross-species, multiple hybridization systems enable genomes to be rapidly assigned to chromosomes [Damas et al., 2017]. A universal set of avian BAC probes has even shown considerable hybridization success on reptile (turtle and lizard) chromosomes [Damas et al., 2017]. It is time to start applying such universal probe sets to as many species as possible with genome assemblies.

Obtaining a more in-depth insight into the mechanisms driving reptile chromosome evolution requires the chromosomal features at evolutionary breakpoints to be determined. At a sequence level, the features for evolutionary breakpoints in mammals and birds include enrichment with repetitive sequences and high gene density, and particularly include genes involved in adaptive processes [reviewed in Farré et al., 2015]. Of course, chromosomes are more than just DNA, and an approach that takes into account the 3D structure of a chromosome is likely to provide a much deeper understanding of reptile chromosome evolution [Farré et al., 2015]. By taking a "chromosomics" [Claussen, 2005] approach, incorporating cytogenetic, genomic, and epigenomic data, we can gain far greater insight into the factors driving the high level of karyotypic diversity among reptiles.

Sex Chromosomes

"The cited observations put an end to a discussion that lasted for a quarter of a century. Morphologically distinguishable sex chromosomes are absent in Reptilia, as is known to be the case also for the Anamnia; the females of Birds and the males of Mammals are heterogametic" [Matthey and van Brink, 1957].

ZZ/ZW sex chromosomes from 2 species of snakes, *Lycodon aulicus* and *Macropisthodon rudis carinatus* were described by Nakamura in 1935 [references in Olmo and Signorino, 2005]. However, the abovementioned statement by Matthey and van Brink [1957] signifies the rarity of sex chromosomes identified in reptiles during the early era of reptile cytogenetics. One of the reasons was the limitation of early cytogenetic techniques to detect small-scale heteromorphy and identify microchromosomes as sex chromosomes, which are now detectable by advanced cytogenetics, such as CGH [Ezaz et al., 2005, 2006; Martinez et al., 2008]. Since then, sex chromosomes have been identified in more than 1,500 reptiles, including many snakes and lizards, and several turtles, displaying enormous diversity in morphologies represented by simple XY and ZW systems as well as by multiple sex chromosomes including XXY and ZZW.

With the great diversity in sex chromosomes and variation in sex determination modes, much of the motivation for studying reptile sex chromosomes has been to determine their evolutionary origin, their gene content, and ultimately to identify master sex-determining genes, although such a gene is yet to be identified for any reptile. Therefore, molecular cytogenetics has been applied extensively to examining reptile sex chromosomes, more so than it has to autosomes.

One of the first studies to identify genes on a reptile sex chromosome mapped cDNA clones onto the chromosomes of the Japanese four-striped rat snake (*Elaphe quadrivirgata*), which demonstrated that the snake Z corresponded to chicken chromosomes 2 and 27, whereas snake chromosome 2 shares homology with the chicken Z [Matsubara et al., 2006]. Furthermore, the 11 *E. quadrivirgata* Z chromosome genes also mapped to the Z chromosomes of 2 other species: the Burmese python (*Python molurus bivittatus*) and the habu (*Trimeresurus* (*Protobothrops*) *flavoviridis*), suggesting that the Z chromosome is conserved across the snake lineage [Matsubara et al., 2006]. Varying levels of differentiation between the Z and W of these 3 species were observed, with all 11 genes mapping to the *P. molurus* W, only 3 were localized to the *E. quadrivirgata* W and none to the *T. flavoviridis* W [Mat-

subara et al., 2006]. Until recently, all snakes were thought to possess this conserved ZZ/ZW system with varying degrees of W differentiation [Ohno, 1967; Matsubara et al., 2006; Vicoso et al., 2013]. Lately, independently evolved XX/XY sex chromosomes have been identified in boa constrictor (*Boa imperator*) and Burmese python (*P. molurus bivittatus*) by using restriction-site-associated DNA sequencing (RAD-seq) to identify sex-specific markers and then mapping these to assembled python or boa genomes [Gamble et al., 2017]. The step missing to complete this approach is to physically map these sex-specific markers back onto chromosomes from these species. Nevertheless, these findings throw into doubt the accepted idea of a stable ZZ/ZW sex chromosome system among snakes and warrant a close reevaluation of the cytogenetic data for this group of reptiles [Gamble et al., 2017].

A combination of chromosome painting and gene mapping has highlighted the diversity of turtle sex chromosomes. For instance, cDNA mapping of 16 chicken Z chromosome genes to *Staurotypus* turtle XY sex chromosomes (Mexican giant musk turtle, *Staurotypus triporcatus;* the giant musk turtle *S. salvinii*) identified syntenic blocks spanning both the short and long arm of chicken Z and the long arm of the emu Z [Kawagoshi et al., 2014], highlighting possible retention of evolutionary conserved synteny between avian ZW and *Stauroptypus* XY sex chromosomes. The Chinese soft-shelled turtle (*P. sinensis*) has a pair of microchromosomes with homology to chicken 15 as a ZZ/ZW sex chromosome system. Of 4 genes localized to the Z chromosome, only 2 mapped to the W, suggesting differentiation of the Z and W chromosomes [Kawagoshi et al., 2009]. In contrast, the XX/XY chromosomes of the black marsh turtle (*Siebenrockiella crassicollis*) and wood turtle (*Glyptemys insculpta*) share homology with chicken chromosome 5 and all clones mapped to both the X and the Y [Kawagoshi et al., 2012; Montiel et al., 2017]. An inversion incorporating the male-specific region of the wood turtle Y chromosome may have been an important step in the divergence of the X and Y and the evolution of GSD in this species [Montiel et al., 2017].

A lack of homology between the avian Z and reptile sex chromosomes was determined to apply more generally across reptiles as a probe for the chicken Z chromosome hybridized to autosomes in species with known sex chromosomes [Pokorná et al., 2011]. A couple of exceptions to this observation include a member of the Gekkonidae family, for which gene mapping localized 6 chicken Z genes to the Z chromosome of the Hokou gecko (*G. hokouensis*) [Kawai et al., 2009]. Z chromosomes of other members of the Gekkonidae family do not share homology with the chicken Z, suggesting that the Z chromosome of *G. hokouensis* is not an ancestral sex chromosome among squamates [Matsubara et al., 2014].

Although there have been a number of in silico studies to infer sex chromosome homologies of reptiles, in particular with chicken [e.g., for review see Ezaz et al., 2017], physical mapping of sex chromosome genes have only been reported for 3 species of snakes, 4 species of turtles, and 4 species of lizards [Matsubara et al., 2006; Kawagoshi et al., 2009, 2012, 2014; Kawai et al., 2009; Alföldi et al., 2011; Ezaz et al., 2013; Srikulnath et al., 2014; Deakin et al., 2016; Montiel et al., 2017]. In these studies, sex chromosome-specific cDNA or BAC clones were isolated by a candidate gene approach and physically mapped to validate their sex chromosomal origin, and their homology to chicken chromosomes was inferred. These studies have identified homologies of reptile XY and ZW sex chromosomes with 9 different chicken chromosomes (2, 5, 6, 9, 15, 17, 23, 27, and ZW) including 2 species of reptiles – a lizard ZW (*G. hokouensis*) and turtle XY (*S. triporcatus* and *S. salvinii*) – sharing homology with chicken ZW chromosomes (Fig. 4) [reviewed in Montiel et al., 2016; Ezaz et al., 2017]. This suggests multiple origins of reptilian sex chromosomes from multiple ancestral proto-sex chromosomes (Fig. 4). However, at least on more than one occasion, the same ancestral autosome has retained its function as a sex chromosome in reptiles and aves (e.g., in chicken, in a gecko, and in a turtle) (Fig. 4). Perhaps this region of the genome contains genes which make it particularly amenable to a role in sex determination and becoming a sex chromosome [Marshall Graves and Peichel, 2010; O'Meally et al., 2012; Montiel et al., 2016; Ezaz et al., 2017]. A better understanding of sex chromosome evolution and the reasons for convergent evolution will emerge if genome sequences are anchored to chromosomes, including sex chromosomes and genes involved in reptile sex determination are identified.

Even when a genome assembly is available, identifying the gene content of the sex chromosomes can be challenging. For instance, determining the gene content of the heteromorphic X and Y microchromosomes of the green anole (*A. carolinesis*) is still not complete, despite this species representing the first reptile genome to be sequenced. Sequencing of an XX female *A. carolinesis* resulted in only a partial assembly (5.3 Mb) of the anole X chromosome [Alföldi et al., 2011]. Genome scaffolds assigned to the X chromosome were homologous to chicken chromosome 15. To extend the amount of X chromosome sequence, orthologs of 38 chicken chromosome 15 genes were as-
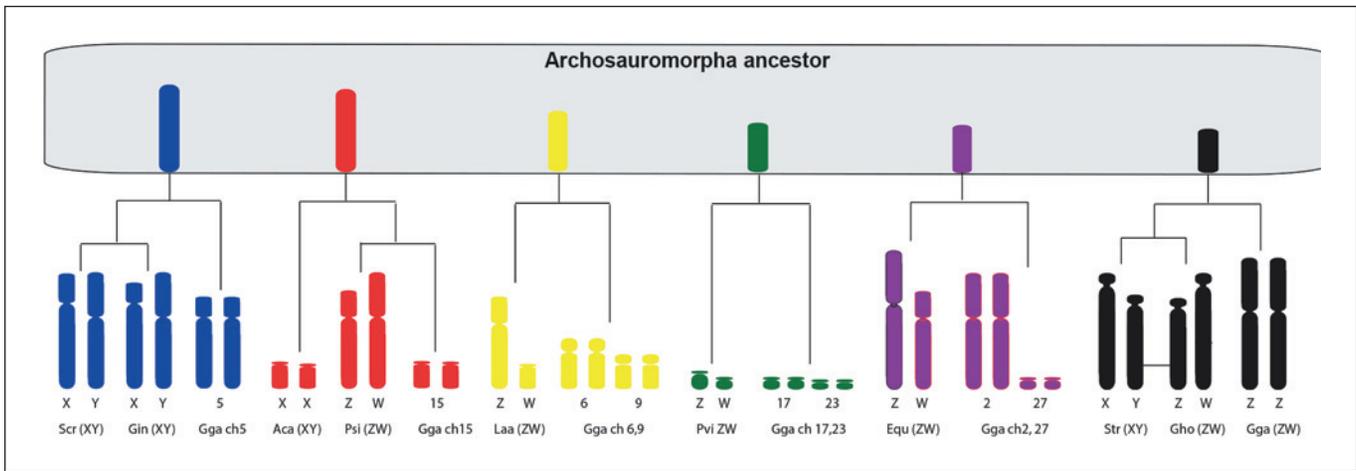
Fig. 4. Reconstruction of reptilian sex chromosome comparisons based on the available physical mapping of cDNA or BAC clones demonstrate multiple origins of sex chromosomes involving at least 6 ancestral proto sex chromosomes. The predicted ancestral karyotypes for the Amniote and Archosauromorpha (crocodiles, dinosaurs, and birds) are based on those previously predicted [Uno et al., 2012]. It is to be noted that predicted homologies are based on very few genes (approximately 50+ genes across 9 reptilian species). Therefore, small-scale chromosome rearrangements could not be predicted conclusively as this would require dense physical mapping. Scr, *Siebenrockiella crassicollis* [Kawagoshi et al., 2012]; Gin, *Glyptemys insculpta* [Montiel et al., 2017]; Gga, *Gallus gallus*; Aca, *Anolis caroliniensis* [Alfoldi et al., 2011]; Psi, *Pelodiscus sinenesis* [Kawagoshi et al., 2009]; Laa, *Lacerta agilis* [Srikulnath et al., 2014]; Pvi, *Pogona vitticeps* [Ezaz et al., 2013; Deakin et al., 2016]; Equ, *Elaphe quadrivirgata* [Matsubara et al., 2006]; Str, *Staurotypus triporcatus* [Kawagoshi et al., 2014]; Gho, *Gekko hokouensis* [Kawai et al., 2009].

signed to the X chromosome using quantitative PCR, where females had twice the dosage of these genes compared to males. This approach more than doubled the number of genes assigned to the anole X, but none appear to be shared with the Y chromosome [Rovatsos et al., 2014]. The only data available for the *A. carolinesis* Y chromosome is a partial sequence of the *Rtdr1y* gene identified using RAD-seq [Gamble and Zarkower, 2014].

A different approach has been taken to sequence the sex chromosomes of an anole species (*A. sagrei*) with heteromorphic X and Y macrochromosomes. In this case, individual X and Y chromosomes were microdissected and sequenced [Kichigin et al., 2016]. The *A. sagrei* X chromosome is homologous to chicken microchromosomes 14, 15, 23, and 28 as well as parts of chicken macrochromosomes 1, 3, 5, and 6. The Y chromosome shares homology with the same chicken chromosomes with the exception of chromosome 15, suggesting that the large X and Y chromosomes of *A. sagrei* were formed from the fusion of several microchromosomes with the ancestral sex chromosome [Kichigin et al., 2016; Giovannotti et al., 2017]. This approach was successful in identifying sequence on the sex chromosomes and is one that may be similarly useful in other species with distinguishable sex chromosomes. Alternatively, individual chromosomes could be microdissected and directly sequenced [Cocca et al., 2015]. Despite the success in obtaining sequence for *A. sagrei* sex chromosomes, a candidate sex-determining gene remains to be identified [Kichigin et al., 2016].

In many species, sex chromosomes are homomorphic, making them even more challenging to identify yet alone sequence. In these cases, CGH was employed as a starting point. An example of the successful use of CGH is the identification of the ZW sex chromosome system in the dragon lizard, *P. vitticeps,* where the sex chromosomes are a pair of microchromosomes [Ezaz et al., 2005]. Once the sex chromosomes are identified, it is then possible to determine the gene content, trace their evolution, and begin the search for a sex-determining gene(s). For *P. vitticeps*, the search for a candidate sex-determining gene began by sequencing a BAC contig surrounding an amplified fragment length polymorphism sex-linked marker, which detected genes sharing homology with chicken chromosome 23, but no candidate sex-determining gene was identified [Ezaz et al., 2013]. Mapping sequence scaffolds from the *P. vitticeps* genome assembly [Georges et al., 2015] assigned 240 genes to the dragon Z chromosome, most of which correspond to chicken chromosome 17 [Deakin et al., 2016]. Among the list of 240 genes is *Nr5a1* (nuclear receptor subfamily 5, group A, member

Deakin/Ezaz

1), which has a known role in vertebrate sex determination and differentiation pathways [Valenzuela et al., 2013], making it a candidate sex-determining gene in this species.

The only other reptile candidate sex-determining gene that has been proposed to date is the *Wt1* gene in the wood turtle (*G. insculpta*) [Montiel et al., 2017]. In this case, CGH identified a macrochromosome XX/XY system, where 3 male-specific regions were detected on the fourth largest macrochromosome pair. An inversion between the X and Y and presumably encompassing *Wt1* (based on *C. picta* genome assembly) led to *Wt1*, another gene involved in the vertebrate sex determination pathway, being proposed as a candidate gene [Montiel et al., 2017]. However, the presence of *Wt1* on *G. insculpta* chromosomes is yet to be validated and has only been inferred from comparisons with *C. picta*.

Molecular cytogenetics has further demonstrated the striking diversity and independent origin of reptile sex chromosomes. In only a few cases has cytogenetics been combined with sequencing to gain more information on the gene content of reptile sex chromosomes [Deakin et al., 2016; Kichigin et al., 2016]. It is only through the combination of cytogenetic and sequencing technologies that we will be able to more fully trace the evolution of reptile sex chromosomes and identify the genes involved in sex determination. Unfortunately, the chromosomes unique to the heterogametic sex (the Y or W) have complex characteristics, with highly repetitive sequence content and unusual chromatin conformation, making them challenging to sequence [Tomaszkiewicz et al., 2017]. As a result, these chromosomes are ignored in most genome sequencing projects, leaving a significant knowledge gap in our understanding of critical aspects of genome evolution and organization. Recently, efforts have been directed to specifically sequence Y or W chromosomes [reviewed in Tomaszkiewicz et al., 2017], yet it is equally important to sequence nondegenerated sex chromosomes (X or Z) to discover molecular triggers that direct differentiation of sex chromosomes leading to degeneration of one of the homologues. It is also important to sequence nondegenerative sex chromosomes for those species with multiple sex chromosomes, identifying alternative molecular mechanisms driving evolution of sex chromosomes via chromosome rearrangements.

Once again, we need to keep in mind that sex chromosomes are dynamic structures and consider the epigenetic status of the sex chromosomes and the genes they contain. For some species of reptiles, examples of sex reversal have been observed, where the genotypic sex is overridden by an environmental cue. One example is *P. vitticeps*, where ZZ eggs incubated at high temperatures develop as phenotypic females [Quinn et al., 2007; Holleley et al., 2015]. In cases such as these, an epigenetic change may be responsible for the sex reversal, a phenomenon observed in the Chinese half-smooth tongue sole fish (*Cynoglossus semilaevis*). In tongue sole ZW embryos exposed to high temperatures, a change in DNA methylation causes an upregulation of the *dmrt1* gene and initiation of the male development pathway (i.e., sex-reversed ZW males) [Shao et al., 2014]. Epigenomic studies could also be important for understanding sex determination in TSD species [Venegas et al., 2016; Radhakrishnan et al., 2017] and the transitions during reptile evolution between TSD and GSD modes. However, good quality genome assemblies are an essential resource for interpreting epigenomic data.

Conclusions

Molecular cytogenetics has provided the first glimpse into reptile chromosome evolution, but a much deeper understanding will be gained by bridging the gap between cytogenetics and genome sequencing. We are now at a stage where the incorporation of cytogenetics with genomics is possible for more species. Moving forward, we need to ensure the closer union of these approaches as well as begin to incorporate epigenomic data in order to decipher the mechanisms responsible for shaping reptile genomes and the remarkably high turnover of sex chromosomes among reptiles.

Disclosure Statement

The authors declare no conflicts of interest.

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