

CHROMOSOME NUMBER AND SEX DETERMINATION COEVOLVE IN TURTLES

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Although much progress has been achieved in understanding the genetic basis of adaptation, the drivers of genome evolution remain obscure. For instance, extensive variation among reptilian genomes continues largely unexplained, yet reptiles hold critical clues about vertebrate evolution. Turtles possess diverse chromosome numbers ($2N = 28\text{--}66$) derived from extensive genomic rearrangements, plus varied sex-determining mechanisms (genotypic and temperature-dependent). Here, we show that rates of evolution in turtle chromosome number are ~20-fold higher along phylogenetic branches where transitions between sex-determining mechanisms also occur, revealing a strong coevolution of these traits and making drift a less likely driver. Directional tests indicate that both traits evolved effectively in synchrony. These events occurred near global extremes in temperature shifts over the last 200 million years, although the role of climate change remains unknown at this point. Two alternative testable explanations for these patterns are proposed. First, selection for sex determination turnover may co-opt mechanisms (e.g., chromatin remodeling) favoring genomic rearrangements. Alternatively, chromosomal rearrangements underlying diploid number evolution may alter gene regulation enabling transitions in sex-determining mechanisms. Our data indicate that the evolution of sex determination is intimately linked to profound genomic changes underlying diploid number evolution, the ecological context of which remains intriguing.

KEY WORDS: Climate change, diploid number, evolution of genome compartmentalization, reptilian vertebrates, sexual development, temperature-dependent and genotypic sex determination.

Understanding how ecology affects genomic structure and function, and mediates their role in the development and evolution of phenotypes, remains an unconquered frontier in evolutionary biology. Answering this fundamental question requires deciphering the proximate and ultimate mechanisms driving genome organization and function, and the relative role of selection and drift in shaping genome evolution (Ellegren 2008; Organ and Shedlock 2009; Valenzuela 2010a). Reptiles exhibit wide variation in diploid number reflective of considerable evolutionary rearrangements of their genome compartmentalization (Olmo 2008). Yet, this diversity has been difficult to explain. Recent Family- and Order-level analyses indicate that karyotypic evolution in reptiles is associated with ecological and morphological diversification, and with compositional genomic differences ([Olmo 2008; Organ et al. 2008], and references

therein). Reptiles also display wide variation in the triggers of the developmental process of sex determination, including male-heterogamety (XX/XY), female-heterogamety (ZZ/ZW), plus environmental (temperature-dependent: TSD) mechanisms (Valenzuela and Lance 2004). Importantly however, it remains unclear whether the evolutionary changes responsible for such genomic and developmental diversity are the result of selective or neutral processes (Valenzuela 2004; Olmo 2008; Organ et al. 2008), and to what extent ecology may have played a significant role.

Remarkably, although the evolution of sex determination appears to be crucially important for the evolution of some key life-history (viviparity) and genomic structural traits (sex chromosomes) (Organ et al. 2009; Valenzuela 2010a), no study has yet examined the coevolution between genome-wide compartmentalization into chromosomes and sex-determining mechanisms

(SDMs). Here, we use a species-level analysis and phylogenetic comparative methods to test for an association between rates of evolution of chromosome number and SDMs in turtles. Turtles serve as an excellent model to address this question because they display extensive differences in diploid number ($2N = 28-66$) (Olmo 2008), and exhibit diverse SDMs, including genotypic (GSD) and environmental (TSD) systems (Valenzuela and Lance 2004).

Materials and Methods

Phylogenetic relationships among turtle taxa were based on the turtle supertree (Iverson et al. 2007) with one polytomy resolved according to a complementary study (Georges and Thomson 2006). The supertree was dated using fossil calibration employing 21 distinct divergence times among major turtle lineages from a previous fossil-calibrated molecular phylogeny (Near et al. 2005), and calibration methods devised for phylogenetic supertrees ([Bininda-Emonds et al. 2007] and references therein). The supertree was then matched to the set of 75 of 314 turtle taxa (Iverson et al. 2007) for which both chromosome number and SDM were available (Fig. 1). This dataset encompassed all known major transitions in SDM in turtles ([Valenzuela and Lance 2004; Martinez et al. 2008], and references therein), and contained species from both suborders of turtles (Cryptodira and Pleurodira), as well as species from all 14 families (with the exception of the monotypic family Platysternidae). This dataset had slightly better representation of Cryptodira as compared to Pleurodira (~26% of all known species vs. 14%), and some lineages (e.g., Kinosternidae) were more densely sampled, as more extensive karyotypic and SDM work had previously been performed on those lineages. However, visual inspection revealed that the species for which data were available were distributed rather evenly throughout the phylogeny of Iverson et al. (2007). Therefore, taxonomic sampling as a result of current data limitations is expected to have a minimal influence on subsequent analyses of evolutionary patterns.

Using this dataset, we estimated ancestral states for SDM for all nodes of the tree, using maximum likelihood procedures (Schluter et al. 1997) implemented in the APE package in R (Paradis 2006). Next, all branches where changes in SDM occurred were identified, and two alternative evolutionary models of chromosomal evolution were evaluated: (A) a model assuming a single rate of chromosome evolution for the entire phylogeny (σ^2), and (B) a model including two rates of chromosome evolution; on branches with and without SDM change (σ_{CHG}^2 vs. $\sigma_{NO\ CHG}^2$; see (Collar et al. 2010) for a similar approach for designing alternative evolutionary models in a phylogenetic context). Rates of chromosome evolution were then estimated using maximum likelihood (O'Meara et al. 2006; Thomas et al. 2006; Revell 2008),

and models A and B were compared using both likelihood ratio tests (LRT) and AICc (Revell 2008). It has recently been noted that estimates of species diversification rates and ancestral character states can be inaccurate, for instance, if a discrete trait is linked to speciation and/or extinction, or if trait evolution has a directional tendency (Maddison et al. 2007; FitzJohn et al. 2009; FitzJohn 2010). Although we acknowledge this possibility, these processes are unlikely to play a major role here, as both GSD and TSD are widespread across the phylogeny and both have evolved multiple times during turtle evolution.

Finally, we used a Bayesian approach implemented with a reversible-jump MCMC algorithm (Pagel and Meade 2006) to compare a model where all transition rates for SDM evolution and chromosome evolution were equal versus models where transitions rates in both SDM and chromosome evolution were allowed to vary. With this approach the preferred model is obtained by comparing model AIC values. Both the ML and Bayesian approaches were implemented in BayesTraits (Pagel et al. 2004).

Results and Discussion

We evaluated two alternative evolutionary models of chromosomal evolution, using a dataset of 75 of 314 turtle taxa for which both chromosome number and SDM were available (Fig. 1) and the phylogenetic relationships among taxa based on the recent supertree for turtles (Iverson et al. 2007) (see Materials and Methods for details). The first model (A) assumed a single rate of chromosome evolution for the entire phylogeny (σ^2), whereas the second model (B) included two rates of chromosome evolution; on branches with and without SDM change respectively (σ_{CHG}^2 vs. $\sigma_{NO\ CHG}^2$).

The two-rate model provided a significantly better explanation for the evolution of chromosome number than did the single rate model: $\ln(A) = -173.40$; $\ln(B) = -150.88$; $LRT = 45.04$; $P_{\chi^2} < 0.00001$; ($AIC_{cA} = 350.97$; $AIC_{cB} = 308.11$). Strikingly, there was a high rate of chromosome evolution on branches where SDM also changed ($\sigma_{CHG}^2 = 1.079$), and very little chromosome evolution on branches where SDM did not change ($\sigma_{NO\ CHG}^2 = 0.054$). These results reveal an increase of nearly 20-fold in the rate of chromosome evolution associated with a change in SDM, and strongly suggest that chromosomal rearrangements and changes in SDMs coevolve in turtles. Further, we found that the rates of chromosome evolution were high on transitional branches regardless of the direction of evolutionary change in SDM ($\sigma_{GSD \rightarrow TSD}^2 = 1.28$; $\sigma_{TSD \rightarrow GSD}^2 = 1.71$). Finally, when these evolutionary events were compared to paleoclimate reconstructions (Zachos et al. 2001; Vieites et al. 2007), we found that they occurred at or near extremes of global temperature shifts experienced over more than 200 million years of turtle evolution, suggesting that climate change may be related to these evolutionary transitions.

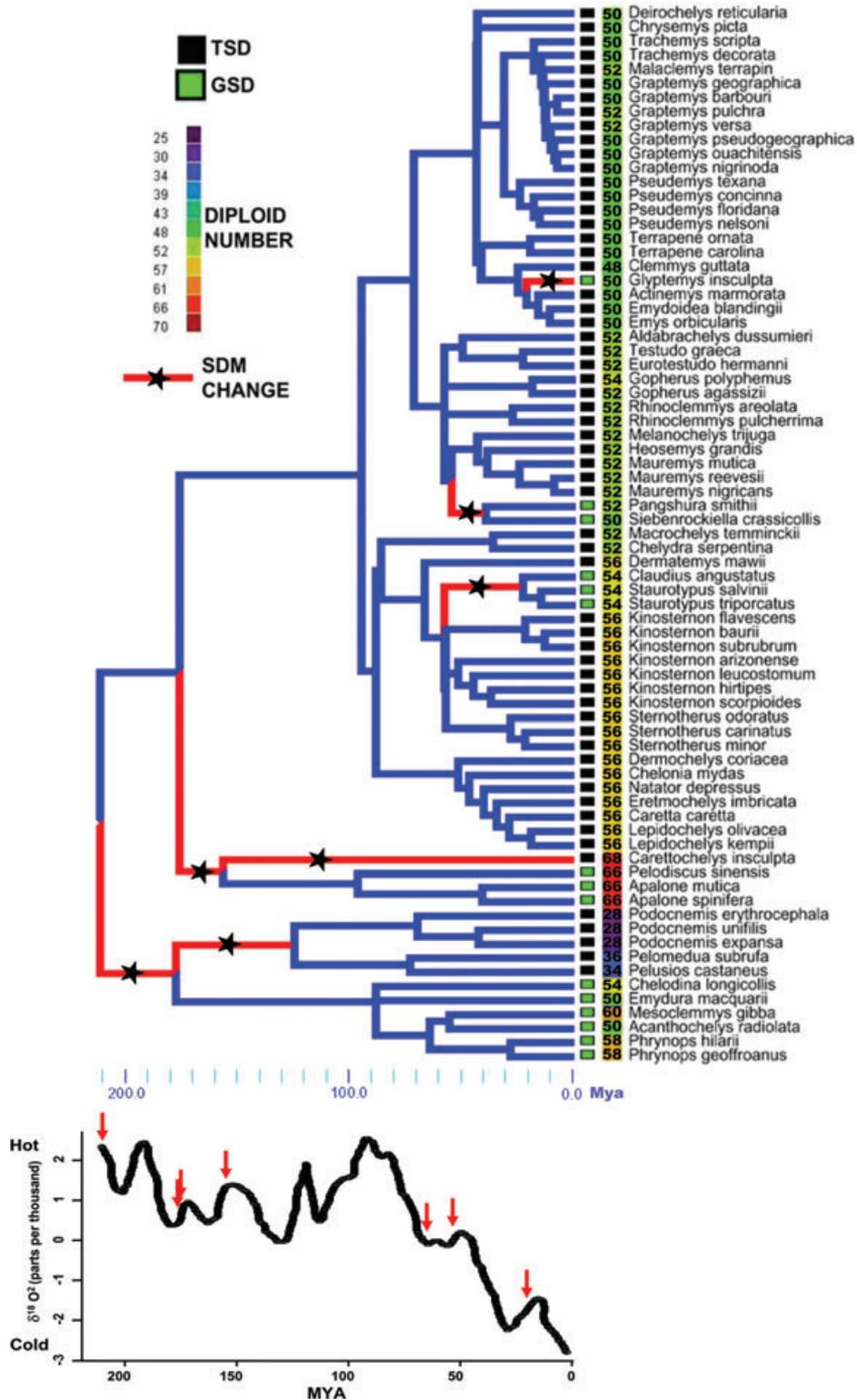


Figure 1. Greater evolutionary changes in chromosome number accompany transitions of sex-determining mechanisms in turtles. Sex-determining mechanism (GSD and TSD) present in extant taxa are indicated as colored rectangles. Red lines with stars indicate branches where transitions among SDM are hypothesized to occur based on maximum likelihood ancestral state estimation. Numbers indicate diploid number per species, color-coded to illustrate evolutionary changes in chromosome number. Branch lengths are proportional to time. Red arrows indicate the approximate timing of transitions in sex-determining mechanism accompanied by high rates of chromosome evolution. These are overlaid on paleoclimate estimates (Vieites et al. 2007) based on deep-sea core oxygen δ^{18} isotope data (Zachos et al. 2001) (redrawn from Vieites et al. 2007).

Given the clear association between evolutionary changes in these two traits, it is of interest to determine whether changes in one trait preceded the other. To address this question we used a maximum likelihood approach (Pagel 1994) to determine the causal directionality of this evolutionary association. Results from this analysis revealed that these traits changed concurrently. Indeed, although chromosome evolution preceded SDM evolution in three of the four possible comparisons of transition rates, this model was not significantly different from a constrained model of equal rates for these transitions ($\ln(L)_{\text{diff.rates}} = -38.5244$; $\ln(L)_{\text{equal.rates}} = -39.8076$; $\chi^2_{df=3} = 2.566$; $P = 0.4634$). Likewise, AIC comparisons revealed that the constrained model was favored ($\text{AIC}_{\text{diff.rates}} = 93.048$; $\text{AIC}_{\text{equal.rates}} = 89.615$; $\Delta\text{AIC} = 3.43$). Further, a Bayesian approach (Pagel and Meade 2006) comparing various models of causality found that the preferred model was one where all transition rates for SDM evolution and chromosome evolution were equal, confirming our ML analysis. Therefore, although there was a strong statistical association between evolutionary shifts in SDM and in chromosome number, the causal directionality of this association could not be determined. This result suggests that sex determination and genome compartmentalization change in such a tight manner that they effectively coevolve in synchrony, rather than one preceding the other. Such a strong pattern of multiple and simultaneous changes in SDM and chromosome number suggests that selection may be the underlying mechanism responsible for these changes (a result consistent with previous theoretical and empirical analyses: (Bull 1983; Valenzuela 2004), although it should be noted that drift cannot be ruled out completely as a possible mechanism.

So how can this coevolutionary synchrony be explained? One possible reason for this correlation is that selective forces driving the evolution of SDMs (Valenzuela 2004) may directly or indirectly favor changes in genomic compartmentalization. For instance, selective forces at the ecological level can influence genomic organization by favoring the evolution of sex chromosomes (Valenzuela 2010a), the most widespread SDM in animals. In the results presented here, all transitions in SDM and their corresponding high rates of chromosome evolution occurred at or near extreme peaks of change in global temperature (Zachos et al. 2001; Vieites et al. 2007); a pattern that suggests a possible relationship between environmental disturbance and evolutionary shifts. Coincidentally, episodes of climate change are associated with other major evolutionary events, such as increased diversification rates in many organisms, including mammals, salamanders, birds, and some invertebrates and angiosperms ([Bininda-Emonds et al. 2007; Vieites et al. 2007], and references therein). It is thus tempting to speculate that not only speciation rates but also life-history and structural genomic traits may evolve in response to selective pressures imposed by abiotic disturbances.

Others have noted that sex determination turnover may be a significant driver for the evolution of other important life-history parameters (Organ et al. 2009). Importantly, evolutionary transitions between GSD to TSD involve an increase in the plasticity of the genome to environmental temperature during embryogenesis whereas transitions from TSD to GSD involved an increase in developmental canalization (Valenzuela 2004). Although such modifications in plasticity have been documented at the level of gene expression (e.g., Valenzuela 2008, 2010b), the master factor responsible for sensing the environmental cue and transducing it into a developmentally plastic response remains elusive. It is possible that such sensory mechanism involves a factor responsible for chromatin remodeling as occurs in other eukaryotes (Kumar and Wigge 2009), which is known to exert control over transcriptional activity (Ho and Crabtree 2010). Notably, chromatin remodeling has also been linked to genome instability and chromosomal rearrangements (Metcalf et al. 2007). Thus, we hypothesize that selection for transitions between sex-determining systems might have favored the recruitment (or adjustment) of a mechanism that induces genome instability (such as chromatin remodeling) enabling an increase in chromosomal fusions and fissions and the ensuing higher rate of change in diploid number on those phylogenetic branches.

An alternative causal explanation can also be contemplated. Changes in genomic structure can be associated with the origin of key evolutionary innovations as well as with adaptation and speciation (Hoffmann and Rieseberg 2008). Thus, it is possible that the chromosomal rearrangements underlying the evolution of diploid number might be the trigger of correlated transitions in SDM. For instance, large chromosomal rearrangements such as inversions may reduce recombination, thus preserving the co-localization in chromosomes (synteny) of groups of adaptive genes in linkage disequilibrium, or promoting speciation by inducing genetic isolation among subpopulations (Hoffmann and Rieseberg 2008). Furthermore, changes in *cis* regulation of gene expression have been linked to critical phenotypic evolution associated with speciation (Prud'homme et al. 2006; Jeong et al. 2008). Importantly, changes in turtle diploid number involve variation in the number of microchromosomes (Olmo 2008). Microchromosomes in turtles and other vertebrates are GC-rich and gene-rich (Federico et al. 2006), have elevated rates of nucleotide substitution (Webster et al. 2006), and in some reptiles contain critical genes for sex determination ([Martinez et al. 2008], and references therein). Interestingly, ultra-long telomere sequences mapped to microchromosomes in chicken are linked to meiotic instability, and may foster genetic variation at chromosomal termini by promoting higher recombination rates, as well as contribute to sex chromosome organization (Rodrigue et al. 2005). Therefore, we hypothesize as an alternative, that the changes in genome compartmentalization that characterize the evolution of diploid number in chelonians

as reported here may have altered syntenic groups resulting in the modification of the regulation of genes involved in sexual development, perhaps via microchromosomal reorganization, and allowing the evolutionary transitions in SDM. Further research is needed to understand the molecular basis of the chromosomal rearrangements that took place during turtle genome and SDM evolution. For instance, the coevolutionary events reported here may depend on certain genomic compositional traits such as the presence and prevalence of transposable elements or the composition and distribution of isochores, which are linked to genome repatterning in vertebrates (Bernardi 1993; Dobigny et al. 2004). Finally, another possibility is that there is no direct causal relationship between the two patterns, and that both chromosome number and SDM respond independently to distinct evolutionary forces. Further studies on the evolution of chromosome organization and genome regulation in turtles are warranted to test these alternative explanations.

In conclusion, our findings imply that seemingly disparate traits, such as chromosome number and SDM, can coevolve in short succession when changes at one level of organization are strong enough to facilitate the evolution of traits at other levels. Further, the occurrence of these coevolutionary events near times of past extreme global temperature shifts is intriguing and worthy of future study in light of current and future climate change predictions. Our study exemplifies how the marriage of evolutionary genomics, developmental biology, and ecology provides fundamental insight into how abiotic and biotic factors might mediate the regulation of gene networks underlying development, and exert selective pressures that drive the evolution of genome function and genome structure, which in turn influences the phenotypic landscape, setting in motion a coevolutionary process across levels of biological organization that we have yet to fully unravel.

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