Molecular evolution of *Dmrt1* accompanies change of sex-determining mechanisms in reptilia

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In reptiles, sex-determining mechanisms have evolved repeatedly and reversibly between genotypic and temperature-dependent sex determination. The gene *Dmrt1* directs male determination in chicken (and presumably other birds), and regulates sex differentiation in animals as distantly related as fruit flies, nematodes and humans. Here, we show a consistent molecular difference in *Dmrt1* between reptiles with genotypic and temperature-dependent sex determination. Among 34 non-avian reptiles, a convergently evolved pair of amino acids encoded by sequence within exon 2 near the DM-binding domain of *Dmrt1* distinguishes species with either type of sex determination. We suggest that this amino acid shift accompanied the evolution of genotypic sex determination from an ancestral condition of temperature-dependent sex determination at least three times among reptiles, as evident in turtles, birds and squamates. This novel hypothesis describes the evolution of sex-determining mechanisms as turnover events accompanied by one or two small mutations.

1. Introduction

Genotypic (GSD) and temperature-dependent sex determination (TSD) have evolved reversibly and repeatedly in reptiles [1,2]. At least four studies have reported reptile phylogenies with varying frequencies of change between GSD and TSD [1–4]. Although sex chromosomes have been retained throughout birds and snakes, lizards exhibit abundant turnover of sex chromosomes and sex-determining systems [5–8]. TSD is attributed to all crocodilians, most turtles and some lizards, and GSD has been inferred for extinct marine reptiles [9].

In this study, we describe an amino acid shift within the protein-coding region of the gene *Dmrt1* that largely distinguishes reptiles characterized as exhibiting either TSD or GSD. *DMRT1* is essential in double dosage for male development in humans, despite its location on an autosome [10] and its functional activation after *Sry*, the master sex-determining gene [11]. *Dmrt1* is directly responsible for the initiation of male sex differentiation in chicken, *Gallus gallus* [12]. This gene is considered the sex-determining gene in chicken, because among genes that govern sexual development, *Dmrt1* resides on the sex chromosomes, acts in a sex-specific pattern in the gonad earliest among

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those genes, and sex is reversed when *Dmrt1* expression is suppressed [12]. In chicken, *Dmrt1* knockdown is followed by decreased activity of Sox9, a gene responsible for testis differentiation, resulting in a female phenotype [12]. Here, we show that two mutated amino acids in *Dmrt1* accompanied a convergent phenotype of GSD at least three times in reptiles.

2. Material and methods

Novel *Dmrt1* sequences were obtained for fourteen reptiles by sequencing targeted PCR products from RNAs and for tuatara, *Sphenodon punctatus*, by searching an unpublished genome database. Other *Dmrt1* sequences were downloaded from GenBank at the National Center for Biotechnology Information [13] or from Ensembl [14] (electronic supplementary material). The amino acid alignment was curated using BioEdit [15] and aligned in Seaview v. 4.5.1 using the Muscle algorithm [16]. The program PRANK was used to align DNA sequences, using the codon option [17].

PrinML v. 3.0 [18] was used to construct the phylogeny of *Dmrt1* using DNA sequences (less than or equal to 733 nt). Topology and branch lengths of the tree were inferred from the *Dmrt1* sequence data using the HKY model. We specified a general time reversible model with gamma-distributed rate variation and invariant sites. We used MEGA v. 6.0 [19] to infer ancestral amino acid sequences at selected nodes using maximum-likelihood and the LG model [20] of amino acid evolution. A time-calibrated tree was created in Mesquite v. 3.01 [21] with the topology from our phylogenetic inference and branch lengths manually adjusted by Date-a-clade [22], using previously published divergence times [23–25].

The BayesTraits program (http://www.evolution.rdg.ac.uk/BayesTraits.html) was used to run a reversible-jump MCMC algorithm on character states for sex-determining mechanism in extant species [9] and ancestral reconstructions of sex-determining mechanisms in the ancestral archosaur, diapsid and lepidosaur [1] to test for correlated evolution between TSD and the combination of threonine at position no. 54 and serine at position no. 57. We used a gamma-distributed hyperprior with mean and variance randomly sampled from 0 to 10. The MCMC ran for 2 100 000 iterations with a 100 000 burn-in and a sampling frequency of 1000. Sex-determining mechanisms were coded as 0 for GSD and 1 for TSD. Sequence data were coded as presence (1) or absence (0) of threonine at position no. 54 and presence (1) or absence (0) of serine at position no. 57.

3. Results

Using the time-calibrated tree, we found evidence that changes from an ancestral TSD state with threonine at position no. 54 (T54) and serine at position no. 57 (S57) of *Dmrt1* (posterior probability (PP) = 95%) are associated with GSD evolution in reptiles. Further, we found 89% posterior support using the tree with branch lengths in units of substitutions per site of *Dmrt1*. Our results also suggest that evolution of T54 was dependent on evolution of S57 (PP = 99.9% using the time-calibrated tree and PP = 95% using the tree with branch lengths in substitutions). Sites 54 and 57 are found within exon 2 near the DM-binding domain of *Dmrt1*. Maximum-likelihood phylogenetic inference supports T54 and S57 in the ancestral archosaur and diapsid and T54 and T57 in the ancestral lepidosaur with probabilities greater than 90% (figure 1). The covariation of these two amino acids and sex-determining mechanism was originally identified by visual inspection of full-length *Dmrt1* sequences across a subset of reptiles. The T54–S57 condition is found in all available *Dmrt1* sequences for extant reptiles with TSD, with the exception of *Pelomedusa subrufa*, *Polocemus expansa*, Carettocelys insculpta and *Eublepharis macularius*. All 18 extant reptiles in our dataset that exhibit GSD, and all nine sampled birds differ in one or both of these sites, suggesting a conformational difference between *Dmrt1* in avian and non-avian reptiles with different sex-determining mechanisms. Low levels of documented homology and partial coverage for *Dmrt1* protein prevented us from characterizing the effect of the two AA shift on three-dimensional conformation of the protein.

4. Discussion

This is the first report, to the best of our knowledge, of a molecular difference in a sex-determining gene between reptiles with GSD or TSD. Several lines of evidence support the idea that this amino acid shift accompanies turnover of sex-determining mechanisms. *Dmrt1* is known to direct sexual development in chicken [12], a species found within Reptilia, a clade known for frequent and reversible changes in sex-determining mechanism [1]. *Dmrt1* is expressed more in gonadal tissues of males than of females, even in a turtle that has TSD [26]. In other species, including mice and rats, *Dmrt1* contributes to male sexual development but does not act first among sex-differentiating genes [27]. Capture or influence by one gene on sexual development could occur at any point in the gene cascade [28]. Therefore, we are not surprised to find exceptions to the two AA pattern across sampled reptiles. Sex-determining mechanisms have changed frequently, suggesting more than one cause. GSD, in extant therian mammals, is driven predominantly by Sry. Amino acid shifts in *Dmrt1* are not likely to alter sex-determining mechanism in those species or in amphibians, all of which have GSD. Nonetheless, the relatively tight correlation of T54–S57 or other amino acid states and sex determination phenotypes, despite shifts in key driver genes, is intriguing. Even as a midpoint in the cascade, change in function of *Dmrt1* could change overall function and thermal sensitivity of the cascade. By this model, in most sampled reptiles with TSD, *Dmrt1* has the T54–S57 condition but still functions as part of the cascade of genes responsible for sexual development, enabling function and avoiding loss by gene conversion.

By using the amino acid shift as a proxy for sex-determining mechanism in a phylogenetic analysis, we replicated ancestral reconstructions that were previously inferred based on characterizations of mechanisms in a family-level analysis [1]. The ancestral lepidosaur is reconstructed as potentially exhibiting T54–T57, predicted to accompany GSD, as inferred using a different criterion. Likewise, the ancestral archosaur and diapsid are reconstructed as exhibiting T54–S57, indicative of TSD, also shown by previous reconstructions [1]. The ancestral amniote was also reconstructed as exhibiting T54–S57 despite lack of robust reconstruction of that ancestor as exhibiting TSD. *Staurotypus triporcatus*, a turtle with XY sex chromosomes homologous to chicken ZW sex chromosomes [29], exhibits an S54–S57 pattern also found in birds (figure 1). Finally, *Carettocelys insculpta*, a turtle with the only inferred reversal from GSD to TSD in chelonians [4], retains the S54–A57 pattern found in its GSD sister family Trionychidae, suggesting that TSD may have re-evolved by a different path from the TS amino acid state in *Carettocelys*. Thus, the TS amino acid state
appears to allow TSD, but TSD is likely to be possible in the absence of the TS state, as GSD is possible in the presence of TS seen in amphibians and mammals. The relationship between our described amino acid shift and sex determination accompanied a series of changes that altered the mechanisms’ thermal sensitivity.

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