**Thermosensitivity profiles of SF1 and WT1 do not predict rate changes in the coding sequences of these genes across turtle species exhibiting TSD and GSD**

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**Introduction**

Thermosensitivity is important for species exhibiting temperature dependent sex determination (TSD), and understanding its role in development is an important, unanswered question. RNA-seq experiments in *Chrysemys picta* (a TSD species) and *Apalone spinifera* (a genotypic sex determination (GSD) species) have revealed interesting thermosensitivity patterns in some genes known to be related to the urogenital networks in mammals (Radhakrishnan *et al*, 2017). TSD is believed to be the ancestral state in turtles, with GSD arising a number of times along with reversions back to TSD (Valenzuela and Adams, 2011).

Transcription factors are important regulatory proteins used in coordination of gene expression. Their binding site recognition across vertebrates has been shown to exhibit high conservation (Schmidt *et al*, 2010). A number of them are known to be important in the vertebrate sex determination network and have showing interesting differential expression patterns in turtles with TSD (Radhakrishnan *et al*, 2017).

Wt1 is a transcription factor involved in urogenital development (GCID: GC11M032365) and exhibits thermosensitivity in both *C. picta* and *A. spinifera* (Radhakrishnan *et al*, 2017), two distantly related *Cryptodiran* turtle species. In *C. picta* it has shown male-biased differential expression during the thermosensitive period (TSP) (developmental stages 19 and 22). The pattern is mixed in *A. spinifera* with male biased expression prior to and late in the TSP (developmental stages 15 and 22) but female biased expression earlier in the TSP (developmental stage 19) (Radhakrishnan *et al*, 2017). This raises the question of why a GSD species from an ancient GSD lineage would still exhibit thermosensitivity in genes proposed to be involved in sex determination?

Sf1 is a transcription factor involved in sex determination (GCID: GC09M124481). Its pattern of thermosensitivity is more as one might expect between TSD and GSD turtles with thermosensitivity present in *C. picta* and absent in *A. spinifera*. It shows male-biased differential expression during the TSP (developmental stages 19 and 22) (Radhakrishnan *et al*, 2017).

How will the evolutionary history of these two transcription factors compare across TSD and GSD species? Both are important for sex determination but one has retained thermosensitivity and another does not. Questions regarding the thermosensitivity patterns of these genes render it worth examining their evolutionary history using phylogenetic approaches.

**Hypotheses**

Given the conserved thermosensitivity patterns observed in Wt1 across *C. picta* and *A. spinifera*, WT1 will exhibit similar evolutionary rates across GSD and TSD species.

Considering the loss of thermosensitivity observed in Sf1 in *A. spinifera* relative to *C. picta*, it is predicted SF1 will exhibit faster evolution in GSD species relative to TSD species.

Testing these hypotheses will provided a preliminary test of whether changes in thermosensitivity is reflected in evolutionary rates of coding sequences. Other transcription factors exhibit similar patterns to the two studied here. While the focus of this project was on these two transcription factors there is no reason the methods could not be applied to others.

Table 1. Species used in this study, abbreviations associated with their analysis (6-letters are used in analysis files only), and corresponding sex determination mechanisms. Outgroups used to root the species tree are in bold.

|  |  |  |
| --- | --- | --- |
| Species Name | Abbreviations | Sex Determining Mechanism |
| *Chrysemys picta* | ChrPic, CPI | TSD |
| *Trachemys scripta* | TraScr, TSC | TSD |
| *Glyptemys insculpta* | GlyIns, GIN | GSD |
| *Staurotypus triporcatus* | StaTri, STR | GSD |
| *Chelonia mydas* | CheMyd, CMY | TSD |
| *Carettochelys insculpta* | CarIns, CIN | TSD |
| *Pelodiscus sinensis* | PelSin, PSI | GSD |
| *Apalone spinifera* | ApaSpi, ASP | GSD |
| *Podocnemis expansa* | PodExp, PEX | TSD |
| *Emydura macquarii* | EmyMac, EMA | GSD |
| ***Alligator mississippiensis*** | **AllMis, AMI** | **TSD** |
| ***Gallus gallus*** | **GalGal, GGA** | **GSD** |

**Methods**

Coding sequences were obtained from a previous study (Literman *et al*, 2017) for ten turtle species and an alligator species for the genes SF1 (also known as NR5A1) and WT1 . Chicken coding sequences for these genes were obtained from the latest NCBI release (103). Separately, sequences were concatenated into a single file and aligned with MAFFT (v7.245) using the --auto parameter for the alignment and --phylipout parameter to produce phylip alignment files. Alignments were tested for differences in rates of evolution between TSD and GSD species for each gene, respectively. Hypotheses for rates of evolution were based off of a published species tree (adapted from Literman *et al*, 2017 and Valenzuela and Adams, 2011). Using CODEML (PAML v4.9), log likelihoods were calculated for the hypothesis that GSD and TSD species would exhibit different rates of evolution for these genes (parameters: runmode=0, CodonFreq=2, model=2, kappa and omega were estimated).

Due to the broad distribution of the two sex determining mechanisms across the species tree for turtles (Fig. 1), species were broken up into four test groups to look for trends in evolutionary rate differences of each gene. This enabled more straightforward labeling of hypothesis trees in such a way that controlled for divergence times of the group. Test groups were taxonomically defined (Fig. 1 and Table 2). In addition to the above analyses, the same kind of test was run comparing rates on the branches hypothesized to contain TSD 🡪 GSD transitions (Fig. 1) (Valenzuela and Adams 2011) to the null that all branches experienced the same rate. CPI and ASP branches were also compared for differences in evolutionary rates due to their being species of interest. CPI and ASP represent two greatly diverged lineages of *Cryptodiran* turtles exhibiting different sex determination mechanisms (TSD and GSD, respectively). Finally, the branches leading to the outgroups were tested for significantly different rates to determine how their inclusion in the alignment may have impacted the results. Above analyses were evaluated for significance using likelihood ratio tests with one degree of freedom.

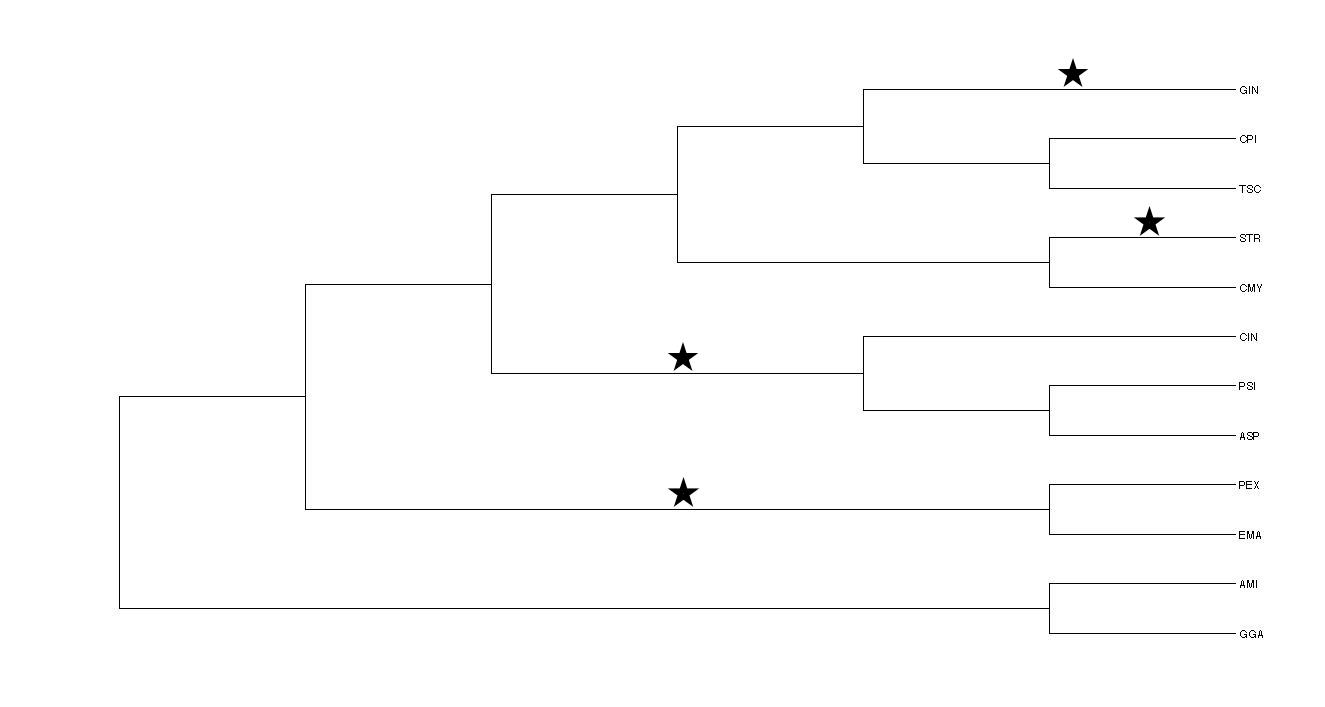


Figure 1. Species tree used in analysis of gene evolution rates (based on Literman et al, 2017). AMI and GGA represent the outgroup. Stars indicate branches where TSD 🡪 GSD transitions have been hypothesized to occur (Valenzuela and Adams 2011). See Table # for name abbreviations. Test groups were as follows: 1-GIN, CPI, TSC; 2-STR, CMY; 3-CIN, PSI, ASP; 4-PEX, EMA. Tree produced in Dendroscope (v3.5.9).

Table 2. Hypotheses tested for SF1 and WT1. Hash marks indicate how branches were labeled for hypotheses of rate variation. Branches were labeled back to the point of divergence for each group or comparison. Trees were unrooted.

|  |  |  |
| --- | --- | --- |
| Test | Alternative Hypothesis | Null Hypothesis |
| Group 1 | #1:CPI, TSC #2:GIN | #1:CPI, TSC, GIN |
| Group 2 | #1:CMY  #2:STR | #1:CMY, STR |
| Group 3 | #1:CIN  #2:PSI, ASP | #1:CIN, PSI, ASP |
| Group 4 | #1:PEX  #2:EMA | #1:PEX, EMA |
| TSD 🡪 GSD | Marked by stars in Fig. 1 | All branches the same |
| CPI vs. ASP | #1: CPI  #2: ASP | #1:CPI, ASP |

**Results**

Likelihood ratio tests (LRT) indicated that among the four subgroups tested, group 4 (PEX vs EMA) had greater maximum likelihood support (p<0.001) for the tree specifying different rates between GSD and TSD species for WT1 (Table 3). The omega value for the GSD branch (EMA) was 0.03633, for the TSD branch (PEX) was 0.32891, and for the remainder of the branches was 0.06428. Additionally the LRT indicated that group 2 (STR vs CMY) had greater maximum likelihood support (p<0.1) for the tree specifying different rates between GSD and TSD species for WT1 (Table 3). The omega value for the GSD branch (STR) was 0.06428, for the TSD branch (CMY) was 0.32891, and for the remainder of the branches was 0.03633. All other results were non-significant for both genes (Tables 3 and 4).

Table 3. Likelihood ratio tests (LRT) for test case hypotheses for the gene WT1. Italics indicates significance of LTR at the p<0.1 level. Bold indicates significance at the p<0.001 level. LnL are log likelihood values produced from CODEML as a part of the PAML program.

|  |  |  |  |
| --- | --- | --- | --- |
| WT1 | Alternate(LnL) | Null(LnL) | LRT |
| Group 1 | -3702.576398 | -3702.576398 | 0.125014 |
| Group 2 | -3702.398412 | -3704.295335 | *3.793846* |
| Group 3 | -3703.338517 | -3704.32346 | 1.969886 |
| Group 4 | -3685.700631 | -3692.447046 | **13.49283** |

Table 4. Likelihood ratio tests (LTR) for test case hypotheses for the gene SF1. LnL are log likelihood values produced from CODEML as a part of the PAML program.

|  |  |  |  |
| --- | --- | --- | --- |
| SF1 | Alternate(LnL) | Null(LnL) | LRT |
| Group 1 | -4870.312772 | -4870.322533 | 0.019522 |
| Group 2 | -4869.72755 | -4870.365022 | 1.274944 |
| Group 3 | -4870.734803 | -4871.920207 | 2.370808 |
| Group 4 | -4871.812956 | -4871.968784 | 0.311656 |

LRT showed significant support for the hypotheses proposing different rates of evolution in TSD🡪GSD transition branches relative to other branches for SF1 but not WT1 (Table 5). The The SF1 omega value for TSD🡪GSD transition branches was 0.07532 and for non-transition branches was 0.04129.

Table 3. Likelihood ratio tests (LTR) for hypothesis that TSD 🡪 GSD transition branches evolve at a faster rate than non-transition branches for the genes SF1 and WT1. LnL are log likelihood values produced from CODEML as a part of the PAML program. Bold indicates significance at the p<0.001 level.

|  |  |  |  |
| --- | --- | --- | --- |
| TSD🡪GSD | Alternate(LnL) | Null(LnL) | LRT |
| SF1 | -4869.801545 | -4872.032349 | **4.461608** |
| WT1 | -3704.241756 | -3704.323549 | 0.163586 |

LRT did not show significant support for greater evolution rates in ASP relative to CPI for either SF1 or WT1 (Table 6).

Table 4. Likelihood ratio tests (LTR) for hypothesis that CPI and ASP branches would exhibit different rates of evolution. LnL are log likelihood values produced from CODEML as a part of the PAML program.

|  |  |  |  |
| --- | --- | --- | --- |
| CPI v. ASP | Alternate(LnL) | Null(LnL) | LRT |
| SF1 | -4871.675995 | -4871.685883 | 0.019776 |
| WT1 | -3702.576398 | -3702.577609 | 0.002422 |

LRT showed significant support for the hypothesis proposing significantly different rates of evolution between the turtle lineages and the outgroup lineage (AMI and GGA) for WT1 but not SF1 (Table 7). The WT1 omega values for turtle branches was 0.12298 and for the outgroup branches was 0.04878.

Table 5. Likelihood ratio tests (LTR) for hypothesis that turtles would exhibit different rates of evolution than outgroup species. LnL are log likelihood values produced from CODEML as a part of the PAML program. Bold indicates significance at the p<0.001 level.

|  |  |  |  |
| --- | --- | --- | --- |
| Turtles v. Outgroups | Alternate(LnL) | Null(LnL) | LRT |
| SF1 | -4871.036181 | -4872.032349 | 1.992336 |
| WT1 | -3697.110981 | -3704.323549 | **14.425136** |

**Discussion**

Results indicate that the rate of evolution of the PEX branch was faster than the EMY branch for WT1. Additionally, there was a trend for the CMY branch rate to be faster than the rate for the STR branch for WT1. Both test cases provide evidence against the hypothesis that GSD species would have faster evolutionary rates than TSD species given that TSD is ancestral. Additionally, these differences were observed in the gene WT1 which is an example of a gene that has exhibited conserved thermosensitivity across two greatly diverged turtle lineages, counter to the prediction that a gene with conserved thermosensitivity would have equal rates of evolution. Contrarily, SF1 (which does not exhibit conserved thermosensitivity) showed no differences in evolutionary rates between any of the lineages tested. This is counter to what was predicted. Indeed, when the CPI vs ASP was tested (the pairing that led to the proposed hypotheses) the LRT did not show support for faster rates in ASP relative to CPI, even though SF1 has exhibited lost thermosensitivity. Testing TSD🡪GSD transitions branches provided conflicting evidence compared to the test groups for SF1. Significant differences in rates of evolution for TSD🡪GSD transition relative to non-transition TSD🡪GSD branches showed a significant difference with transition branches having faster rates. This is evidence in support of the hypothesis of GSD species having faster rates of evolution for SF1. Taken together, results are somewhat inconclusive regarding SF1, however the test on the transition branches is a more appropriate means of examining the question since the branches represent when changes in sex determination mechanism are actually believed to occurred.

Results comparing the rates of evolution of outgroup species relative to turtle species indicated that these lineages did not have different evolutionary rates for SF1, but were slower for WT1. Therefore including these species in the alignment should not have affected results for SF1. The results for WT1 may have been impacted as AMI and GGA were always included in the background group relative to the species being tested. Their slower rate may have lowered the background rate of the tree. This could have dramatized differences between the background and test group, however the null hypothesis tree should have helped control for this discrepancy.

It is possible there is a large constraint on the evolution of these genes as they are important for sex determination (Literman *et al*, 2017). It is also possible that coding sequences are not the best region in which to test these hypotheses. Coding sequences are more likely to be under strong purifying selection because of the potential importance of a gene product’s function. Perhaps promoter sequence would better explain the patterns of thermosensitivity observed in these turtles. Due to turtles being non-model organisms, obtaining promoter sequences is much more difficult than coding sequences.

The observed differences present within test group comparisons could also be due to greater relative divergence between these two species relative to other groupings as test group sets are not comparable because they are not controlled for divergence times. Using test groups (as was done here) is perhaps not the most desirable way to test for differences based on sex determination mechanisms as it divides up the data, limiting the power of the analysis. The method was employed in order to control for divergence times between compared species and in an attempt to show trends across multiple lineages that consistently corresponded to changes in sex determination mechanisms. This was somewhat observed in the results with WT1 showing trends of faster rates in TSD relative to GSD species for two test groups as well as showing no significant difference in branches that had transitions to GSD. Together these results suggest that the coding sequence of WT1 does not experience elevated rates of evolution in a GSD context in turtles.

It is important to note that this was a gene approach rather than a species approach. Individual genes can exhibit evolutionary history that is different from that of the species as a whole. While the gene history itself can be interesting to understand how numerous small scale changes occurred, results on a single gene should not be extrapolated to species-level patterns. Literman *et al* (2017) took a species approach to the question of evolution rates and sex determination transitions by examining many more genes (including SF1 and WT1) and found that sex determination genes in general do not exhibit consistent evolutionary patterns. This is consistent with the findings found here. While results do not provide any consistent evolutionary relationships consistent with thermosensitivity patterns they do provide insight into how these important sex determination genes have evolved across diverse turtle lineages and suggest faster evolutionary rates in TSD rather than GSD turtles. This pattern was also observed in Literman *et al* (2017) which found GSD to TSD reversions to exhibit elevated evolutionary rates. Perhaps the nature of TSD experiences unique selection pressures, a question worthy of further future exploration.

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