



Phylogenomic analyses resolve relationships among garter snakes (*Thamnophis*: Natricinae: Colubridae) and elucidate biogeographic history and morphological evolution

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ABSTRACT

Garter snakes (*Thamnophis*) are a successful group of natricines endemic to North America. They have become important natural models for ecological and evolutionary research, yet prior efforts to resolve phylogenetic relationships have resulted in conflicting topologies and weak support for certain relationships. Here, we use genomic data generated with a reduced representation double-digest RADseq approach to reassess evolutionary relationships across *Thamnophis*. We then use the resulting phylogeny to better understand how biogeography and feeding ecology have influenced lineage diversification and morphological evolution. We recovered highly congruent and strongly supported topologies from maximum likelihood and Bayesian analyses, but some discordance with a multispecies coalescent approach. All phylogenomic estimates split *Thamnophis* into two clades largely defined by northern and southern North American species. Divergence time estimates and biogeographic analyses indicate a mid-Miocene origin of *Thamnophis* in Mexico. In addition, historic vicariant events thought to explain biogeographic patterns in other lineages (e.g., Isthmus of Tehuantepec, Rocky Mountain Range, and Trans-Mexican Volcanic Belt) appear to have influenced patterns of diversification in *Thamnophis* as well. Analyses of morphological traits associated with feeding ecology showed moderate to strong phylogenetic signal. Nevertheless, phylogenetic ANOVA suggested significant differences in certain cranial morphologies between aquatic specialists and garter snakes that are terrestrial-aquatic generalists, independent of evolutionary history. Our new estimate of *Thamnophis* phylogeny yields an improved understanding of the biogeographic history and morphological evolution of garter snakes, and provides a robust framework for future research on these snakes.

1. Introduction

Garter snakes (*Thamnophis*) are among the most familiar and ubiquitous snakes in North America. The roughly 35 species (Rossman et al., 1996; Rossman and Burbrink, 2005; Wood et al., 2011) of this natricine (Colubridae) genus are distributed across most of North America. They range further north than any other reptile in the western hemisphere, occurring from southeast Alaska and the lower Northwest Territories in Canada to as far south as Costa Rica and Panama (Rossman et al., 1996; Stebbins, 2003; Fig. 1). As diurnal, actively foraging snakes that can be locally abundant and are generally associated with aquatic systems and mesic habitats, they are commonly encountered. To many people, they are instantly recognizable as swift, slender snakes, with bold and bright

stripes running down the length of the body.

Thamnophis display remarkable ecological, behavioral, physiological and morphological variation, and even within species at fine spatial scales (e.g., Arnold, 1977; Brodie, 1989; Bronikowski, 2000). These factors, in addition to their relative ease of study in both natural and captive conditions, have made *Thamnophis* model organisms for a range of topics. For example, garter snakes have been instrumental in improving our understanding of quantitative genetics (e.g., Arnold and Phillips, 1999; Brodie, 1993; Garland, 1988), the molecular basis of adaptive traits (e.g., Geffeney et al., 2005; McGlothlin et al., 2014), patterns of coevolution (e.g., Brodie and Brodie, 1999; Hague et al., 2020; Reimche et al., 2020), convergence (e.g., Feldman et al., 2012; McGlothlin et al., 2016), chemical ecology (e.g., Lutterschmidt and

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Maine, 2014; Mason, 1993), feeding ecology (e.g., Britt and Bennett, 2008; Drummond and Burghardt, 1983; Kephart and Arnold, 1982), and life history evolution (e.g. Bronikowski, 2000; Gregory and Skebo, 1998; Robert and Bronikowski, 2010; Shine et al., 2001).

Thamnophis are well suited for examining the influence of geography, ecology, and history on patterns of diversification. The group constitutes a relatively species rich clade that inhabits a wide array of ecological communities and environments across much of North America (Fig. 1). In particular, a host of *Thamnophis* species occupy biodiversity hotspots such as the California Floristic Province (CFP) and Mexican Transition Zone (MTZ) (Mastretta-Yanes et al., 2015; Myers et al., 2000), or span a range of ecoregions and biogeographic boundaries (Rossman et al., 1996; Stebbins, 2003). Such landscape features, along with historical events, are thought to have driven patterns of differentiation within several garter snake species (de Queiroz and Lawson, 2008; Hallas et al., 2021; Ridenhour et al., 2007; Wood et al., 2011). However, we lack a deeper understanding of the role that geography and history have played in diversification across the phylogeny of *Thamnophis* (de Queiroz et al., 2002).

Finally, garter snakes exhibit tremendous dietary variation, with examples including populations that specialize on noxious slugs (Fox, 1951), consume deadly newts (Brodie et al., 2005; Brodie, 1968; Greene and Feldman, 2009), or use venom to subdue mice (Vest, 1981), as well as generalists with among the broadest diets of all North American snakes (Rossman et al. 1996). In fact, a good deal of literature on *Thamnophis* concerns the causes and consequences of this variation in feeding ecology (e.g., Arnold, 1981; Drummond, 1983; Ersan et al., 2020; Manjarrez et al., 2017; Reguera et al., 2011). In addition, specialized behaviors and particular morphological traits are thought to be associated with certain diets and foraging modes in natricine snakes (de Queiroz, 2003; Drummond, 1983; Hampton, 2011; Herrel et al., 2008; Manjarrez et al., 2017; Naumov et al., 2011; Vincent et al., 2009). Nevertheless, a resolved phylogeny is needed to understand the evolution of diet and attendant traits in garter snakes.

Early work to understand relationships among members of *Thamnophis* centered around meristic traits (such as scale variation) and color patterns (Cope, 1892; Fitch, 1948, 1940; Fox, 1951; Rossman and Stewart, 1987; Ruthven, 1908; Van Denburgh and Slevin, 1918). However, many species display extensive geographic variation and regional polymorphism, leading to numerous taxonomic arrangements and rearrangements (reviewed in Rossman et al., 1996). More recently, formal molecular phylogenetic analyses have improved our understanding of diversification and relationships among *Thamnophis* (Alfaro and Arnold, 2001; de Queiroz et al., 2002; de Queiroz and Lawson, 1994; Guo et al., 2012; Lawson and Dessauer, 1979; McVay et al., 2015). Nonetheless, all efforts to date have been based on small numbers of traditional mitochondrial (mtDNA) and nuclear (nDNA) markers, leading to poor resolution for many nodes, and incongruence among analyses (Alfaro and Arnold, 2001; de Queiroz et al., 2002; de Queiroz and Lawson, 1994; McVay et al., 2015). These issues may stem from insufficient variation, incomplete lineage sorting, and perhaps even introgression (de Queiroz et al., 2002; de Queiroz and Lawson, 1994). More fully resolved phylogenetic hypotheses based on larger amounts of data should improve inferences of the biogeographic mechanisms of diversification as well as broader comparative studies of trait evolution.

In recent years, high-throughput sequencing efforts have allowed the application of genome-wide data to phylogenetic analyses, consistently improving phylogenetic estimates and resolving previously troublesome nodes. While phylogenetic analyses based on whole genomes (e.g., Kimball et al. 2019), whole exomes (e.g., Johnson et al., 2018; Knutson et al., 2020; Smith et al., 2011), or targeted enrichment (e.g., ultra-conserved elements, Crawford et al., 2014; Faircloth et al., 2015) demand genomic resources, reduced representation approaches (e.g., restriction site-associated DNA sequencing (RADseq), genotyping by sequencing (GBS)) do not require prior genomic information. Here, we used a double-digest RADseq (ddRADseq) approach to estimate phylogenomic relationships among *Thamnophis* using three complementary phylogenetic approaches: i) maximum likelihood (ML), ii) Bayesian

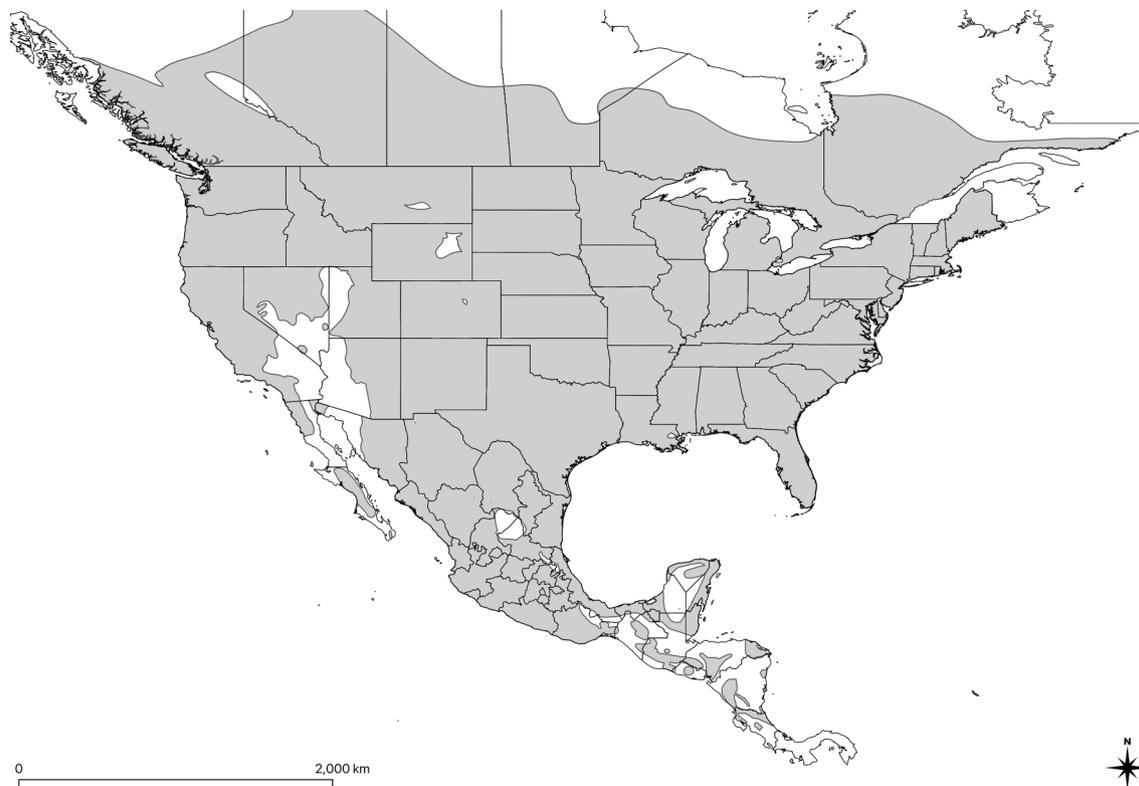


Fig. 1. Summary distribution of all garter snake species (*Thamnophis*) across Nearctic and Neotropical regions (after Rossman et al., 1996; data from IUCN Red List of Threatened Species, 2021).

inference (BI), and iii) multispecies coalescent (MSC). We generated fossil calibrated divergence time estimates and inferred the biogeographic history of the group to assess patterns of lineage diversification associated with dispersal and vicariance events. Lastly, we quantified phylogenetic signal in the evolution of morphological traits associated with feeding strategy and foraging mode across the phylogeny to test notions about cranial evolution in natricines (contrasting trait evolution between aquatic specialists and terrestrial-aquatic generalists). Our overarching goal was to create a robust phylogeny for testing ecological and evolutionary hypotheses, and to provide an evolutionary framework for diverse research focusing on this group.

2. Materials and methods

2.1. Taxonomic sampling

We acquired 43 tissue samples representing 30 of the 35 *Thamnophis* species from natural history museums or personal collection efforts (Table A.1). Because some species remain scarce in collections, or lack suitable tissue samples, we were unable to include *T. linei*, *T. mendax*, *T. postremus*, *T. rossmani*, and *T. unilabialis*. We also obtained tissues from other key natricine taxa (*Clonophis*, *Natrix*, *Nerodia*, *Storeria*, *Tropidoclonion*, and *Virginia*) for use as outgroups, which previous phylogenetic estimates have recovered as closely related to *Thamnophis* (e.g., McVay et al., 2015). Lastly, we included *Adelophis foxi* because this taxon has been recovered within or closely related to *Thamnophis* (de Queiroz et al., 2002; Guo et al., 2012; McVay et al., 2015; McVay and Carstens, 2013).

2.2. ddRADseq data collection

We extracted genomic DNA from either liver or muscle tissues using Qiagen DNeasy Blood & Tissue kits (Qiagen, Inc.; Valencia, CA). We generated sequencing data with a reduced-representation double-digest RADseq approach (ddRADseq; Parchman et al., 2012; Peterson et al., 2012). Such approaches are well suited to phylogenetic inference because they produce large numbers of phylogenetically informative SNPs without the need for prior genomic information (Leaché and Oaks, 2017; Lemmon and Lemmon, 2013; Ree and Hipp, 2015). We first digested genomic DNA with the restriction enzymes *EcoRI* (6-base recognition site) and *MseI* (4-base recognition site). We subsequently used T4 ligase to attach standard Illumina adaptors to the *MseI* restriction sites and Illumina adaptors modified with identifier barcodes (8, 9, or 10 bp in length) to the *EcoRI* restriction sites. All barcode sequences differed by a minimum of three bases so that one or two base sequencing errors within them could be corrected in the sequencing data. We used a high-fidelity proofreading polymerase (iProof polymerase, BioRAD; Hercules, CA) and Illumina primers to amplify barcoded fragments. We pooled libraries and quality screened them on a BioAnalyzer (Agilent, Inc.; Santa Clara, CA), performed size selection (350-450 bp) with a Pippin Prep device (Sage science, Inc.; Beverly, MA), and sequenced on a single Illumina HiSeq 2500 lane at the University of Wisconsin Madison Biotechnology Center.

2.3. Demultiplexing, filtering, and alignment

We first filtered raw sequences for common contaminants (i.e. PhiX, *E. coli*, or Illumina oligos) using *bowtie2* v2.2.5 (Langmead and Salzberg, 2012) and scripts designed for the filtering of raw Illumina data (<https://github.com/ncgr/tapioca>). We then demultiplexed the barcoded reads to individual sample using a Perl script that corrects for ≤ 2 base sequencing errors in barcodes. Filtered fastq files for each sample are available at DRYAD (<https://doi.org/10.5061/dryad.h18931zm7>).

We processed demultiplexed reads into sequence alignments using the *de novo* assembly method in *ipyrad* v0.9.54 (Eaton and Overcast, 2020). We generated two alignments that contained either 20% (M20)

or 30% (M30) missing data to account for its effect on our phylogenomic estimates (Leaché and Oaks, 2017; Lemmon and Lemmon, 2013). Both alignments were constructed with identical parameters except for the minimum samples per locus parameter. The parameters for max low-quality bases and phred Qscore offset were set to five and 33, respectively. Due to the unevenness of coverage depth, we set the minimum depth statistical parameter to six and the minimum depth majority rule parameter to five. Loci that exceeded a depth of 8000 were excluded from alignments to guard against the potential misassembly of paralogous loci. We set a threshold of 0.85 for clustering similarity, and excluded reads with greater than 0.05 uncalled bases, 0.08 heterozygous sites per locus, and 0.5 indels per locus.

2.4. Phylogenomic estimates

We estimated phylogenetic trees using both the M20 and M30 concatenated alignments with ML and BI. In addition, we also utilized unlinked SNPs in an MSC approach. Our ML estimate was generated in *RAxML* v8.2.12 (Stamatakis, 2006) using the “-f a” option, which simultaneously conducts a rapid bootstrapping analysis and searches for the best-scoring tree. Searches were run using the GTR + GAMMA evolutionary model. The number of appropriate bootstrap replicates was determined using the “autoMRE” option. This method evaluates convergence of bootstraps by implementing a *posteriori* bootstrapping analysis using the majority-rule consensus tree criterion, which determined 50 bootstrap replicates as appropriate. Nodes with bootstrap values ≥ 70 were considered significantly supported (Alfaro et al., 2003; Hillis and Bull, 1993).

For our BI estimate, we used *ExaBayes* v1.5.1 (Aberer et al., 2014). We first executed preliminary *ExaBayes* runs to optimize sampling parameters by monitoring average split frequency (ASDSF) between trees, effective sampling size (ESS) values, potential scale reduction factor (PSRF), and convergence between runs as visualized in *Tracer* v1.7.1 (Rambaut et al., 2018). Because of the computational requirements, we were only able to estimate BI relationships of *Thamnophis* for our M20 dataset by executing two individual runs with the *Yggdrasil* command for 700,000 MCMC generations each. This analysis consisted of two coupled chains sampled every 500th generation with a burn-in of 25%. As with preliminary runs, ESS values (≥ 200), ASDSF between trees (≤ 0.01), PSRF (close to 1), and convergence between runs were evaluated. Posterior probabilities ≥ 0.95 were considered as significant support for relationships (Alfaro and Holder, 2006).

We inferred a species tree using quartet sampling under the MSC model (reviewed in Edwards et al., 2016; Liu et al., 2019) employed in *tetrad* v0.9.13, which is similar to the quartet puzzling method implemented in *SVDquartets* (Chifman and Kubatko, 2014). *tetrad* maximizes the number of unlinked SNPs from the *.snps.hdf5* file generated in *ipyrad*. Unlike other methods that take a two-step approach by first generating many individual gene trees and then summarizing the resulting gene trees to infer the species tree (e.g., *ASTRAL* [Zhang et al., 2018], *MP-EST* [Liu et al., 2010], or *NJ_{st}* [Liu and Yu, 2011]), *tetrad* utilizes unlinked SNPs from aligned sequence data to generate quartets under a coalescent model and then uses the *wQMC* algorithm to join the resulting quartets into a species tree. We evaluated all possible quartets and support of relationships was assessed with 50 non-parametric bootstrap replicates.

2.5. Molecular dating

To estimate divergence times among lineages we used *BEAST* v1.10.4. We used our M30 ML phylogenomic dataset for this analysis because this tree is based on the greatest number of informative characters, and the ML and BI topologies generated with both data sets were strongly congruent (see Results). Because of the size of our M30 dataset, we limited sampling to a single representative for each species and then

randomly subsampled 2,000 loci for this analysis to limit biases in our dataset (Huang and Lacey Knowles, 2016; Ree and Hipp, 2015; Villaverde et al., 2021). We converted the .loci file from ipyrad into a matrix of sequences using the `rad2mat` function in the R package RADami (Hipp, 2014; <https://github.com/andrew-hipp/RADami>), and then subsampled loci using a modified custom R script (Villaverde et al., 2021) available on DRYAD (<https://doi.org/10.5061/dryad.h18931zm7>). Finally, we converted the subsampled data matrix to nexus format using the `rad2nex` function in the RADami R package.

We estimated phylogeny and divergence times using the GTR + GAMMA substitution model with estimated base frequencies, and an uncorrelated relaxed clock. We constrained the topology to that of our ML analysis and used a birth-death process speciation model. Fossil data for *Thamnophis* places the origin of the genus in the Barstovian (Holman, 2000). Because the Barstovian has a fairly narrow range (15.97–13.6 Mya), we decided to incorporate more uncertainty in our estimate by calibrating the TMRCA of the genus from the mid to early Miocene [12.44–20.26 Mya; lognormal mean = 16; standard deviation = 2]. We also used the divergence time estimate from Guo et al. (2012) to calibrate the root of our topology that includes *Natrix*, which they recovered to be 19–28 Mya [lognormal mean = 23; standard deviation = 2.378]. Our analysis had a chain length 1×10^8 , and we saved every 10,000th state. Tracer v1.7.1 (Rambaut et al., 2018) was used to verify proper mixing and ESS values. We discarded the first 1×10^7 states as burnin and summarized trees to generate the maximum clade credibility tree using TreeAnnotator v1.10.4.

2.6. Ancestral range reconstruction

To infer the potential influence of biogeographic features on diversification, we conducted an ancestral range reconstruction (ARR) analysis using the Bayesian binary Markov chain Monte Carlo (BBM) approach in RASP v4.0 (Reconstruct Ancestral State in Phylogenies; Yu et al., 2015). As above, we used our M30 ML phylogenomic estimate for this analysis. We pruned outgroups from this tree and limited sampling to a single representative for each species using APE v5.2 (Paradis and Schliep, 2019). Ranges of all included species (Fig. 1) come from the IUCN Red List of Threatened Species (2021) and we verified these from literature (Rossman et al., 1996; Stebbins, 2003). To simplify our biogeographic analysis, we grouped ecoregions (Omernik and Griffith, 2014) into larger units that encompassed the major biomes of North America in a similar manner as Blair and Sánchez-Ramírez (2016; Table A.2), and assigned these units to each species. We chose ecoregions because these units reflect major abiotic and biotic differences between ecological communities and have been useful in ecological models, as well as in conservation and management (Di Marco et al., 2018; Dinerstein et al., 2017; Olson et al., 2001; Olson and Dinerstein, 2002). We set our BBM parameters to default (temperature = 0.1; model = JC; and among-site rate variation = equal), and ran 10 MCMC chains for 1×10^7 generations sampling every 1000 generations after a 25% burn-in. We set the max number of ancestral ranges to six, which represented the largest number of units spanned by any species in our analyses (*T. cyrtopsis* and *T. marcianus*).

2.7. Ancestral state reconstruction, phylogenetic signal, and trait evolution

In snakes, head shape is generally associated with feeding ecology and habitat use, but also with phylogeny (Greene, 1997). We examined the evolution of morphological variation in head shape potentially associated with feeding ecologies and diet composition in natricines (e.g. Bilcke et al., 2006; Hampton, 2011; Herrel et al., 2008). We examined three head traits thought to be important in aquatic or terrestrial foraging strategies in *Thamnophis* (Rossman et al., 1996): i) relative head length (the length of the head divided by snout vent length); ii) relative muzzle length (length of the muzzle divided by frontal length) and; iii)

relative muzzle width (internasorostral contact divided by nasorostral contact). We obtained these data using published information on head measures for each taxon (Rossman et al., 1996; Rossman and Burbrink, 2005; Table A.3). We used relative measures because of the wide variation in overall body sizes among *Thamnophis*, including age related size differences and tremendous sexual size dimorphism. We averaged values in instances of sexually dimorphic characters or variation among subspecies. We then conducted ancestral state reconstruction (ASR) analyses on each of these head traits across the *Thamnophis* phylogeny.

To perform ASR, we used the ML `fastAnc` method implemented with the `contMap` function in the `phytools` R package (Revell, 2012). This ML method estimates character evolution according to Brownian motion (BM; Felsenstein, 1985), and allows for the use of continuous characters. To be consistent with the ARR analysis above, we used the same pruned topology of the M30 ML phylogenomic estimate, although we excluded *Adelophis foxi* due to insufficient morphological data for that species.

We assessed phylogenetic signal for each trait by calculating both Pagel's lambda (λ , Pagel, 1993) and Blomberg's *K* (Blomberg et al., 2003) using the function `phylosig` in `phytools`. These measures evaluate the phylogenetic signal of continuous characters (reviewed in Kamilar and Cooper, 2013; Münkemüller et al., 2012). Both λ and *K* assume a BM model of trait evolution to assess phylogenetic signal. Values of λ and *K* closer to 0 indicate trait evolution independent of phylogeny (no phylogenetic signal), while values of λ and *K* closer to 1 suggest strong phylogenetic signal. In the case of *K*, values > 1 represent patterns of constrained evolution where closely related species are more similar than expected under the BM model (note that λ only varies from 0 to 1).

Finally, we explicitly tested the hypothesis that aquatic specialization places unique functional demands on the cranial morphology of snakes (e.g., Hampton, 2011; Segall et al., 2019, 2016; Vincent et al., 2009). Specifically, we expect garter snakes that forage and feed almost entirely in water to possess relatively longer and narrower heads that improve locomotor, striking, and feeding efficiency in aquatic environments. This is in contrast to garter snakes that are generalists or more terrestrial, and thus under different selective pressures (e.g., Fabre et al., 2016; Hampton, 2011). We categorized *Thamnophis* species as either “aquatic specialists” or “terrestrial-aquatic generalists” following Drummond (1983) and de Queiroz (2003). These categories are based on diet (Table A.4) and foraging behavior, including the proportion of time spent hunting in water, crawling on underwater substrates and into underwater crevices, the proportion of prey strikes underwater, and other specialized behaviors such as the mode of aquatic pursuit and type of aquatic strikes (Drummond, 1983; de Queiroz, 2003). Using these criteria, we considered *T. atratus*, *T. couchii*, *T. gigas*, *T. hammondii*, *T. melanogaster*, *T. nigronuchalis*, *T. rufipunctatus*, and *T. validus* to be aquatic specialists, and grouped all the remaining species as terrestrial-aquatic generalists. Considering all other *Thamnophis* as terrestrial-aquatic generalists may be imperfect, as many taxa could be partitioned into other categories based on diet, terrestrial habits and other aspects of feeding ecology (e.g., some species specialize on terrestrial invertebrates, others forage off the ground in vegetation). However, further subdivision would reduce statistical power and likely require more quantitative information on the diets of each species (outside the scope of this study), and we were primarily interested in the unique demands imposed by a highly aquatic lifestyle.

We then conducted a phylogenetic analysis of variance (phylANOVA) to test whether the three morphological traits differ significantly between aquatic specialists and terrestrial-aquatic generalists, despite potential phylogenetic signal. We used the `phylANOVA` function in the `phytools` R package, and ran each phylANOVA with 1000 simulations for each trait, using our time-calibrated phylogenomic estimate. We then visualized the results of these analyses on calibrated phenograms for each morphological trait in `phytools` using the function `phenogram`.

3. Results

3.1. Assembly of ddRADseq data

After contaminant filtering and barcode demultiplexing, we retained 203,840,318 of 281,355,635 reads generated by the HiSeq 2500 instrument. There was a noticeable 45.6% difference in the number of loci retained between our two filtering and quality control procedures in ipyrad. We retained 22,289 loci for the M20 dataset and 48,867 loci for our M30 dataset. The M20 dataset consisted of 17.02% missing sites, and a 1,939,142 DNA sequence character matrix with 92,244 parsimony informative sites, while the M30 dataset consisted of 23.48% missing sites, resulting in a 4,245,257 DNA sequence character matrix with 198,695 parsimony informative sites.

3.2. Maximum likelihood and Bayesian inference phylogenomic estimates

There was strong congruence between our ML and BI phylogenetic estimates regardless of the amount of missing data between M20 and M30 datasets (Fig. 2A; Figs. A.1-A.2). There was also significant bootstrap (bs) and posterior probability (pp) support for a high proportion of relationships recovered across both ML and BL analyses. Independent runs of ExaBayes converged with 0.001% ASDSF. In addition, ESS values from our combined run exceeded 200, ranging from 254 to 2,100, and PSRF values fell within 1.00–1.01 (Table A.5).

Phylogenomic estimates for each dataset recovered garter snakes as paraphyletic with respect to *Adelophis foxi*, which was nested within

Thamnophis. This clade of *Thamnophis* and *Adelophis* (hereafter referred to as *Thamnophis* for simplicity) was sister to a clade of small terrestrial natricines including *Storeria occipitomaculata* and the semifossorial snakes *Clonophis kirtlandii* and *Virginia striatula*. The water snake *Nerodia cyclopiion* and lined snake *Tropidoclonion lineatum* formed a small clade sister to the remaining ingroup taxa.

The genus *Thamnophis* was divided into two clades containing species whose distributions largely span northern or southern North America. However, *T. eques*, *T. marcianus*, and *T. proximus*, whose distributions extend into Mexico and other parts of southern North America, were inferred to occur in the northern clade. In the northern clade, the ribbon snakes, *T. proximus* and *T. sauritus*, are sister to the widespread *T. sirtalis*. *Thamnophis sirtalis* bifurcated into eastern and western lineages, which were more differentiated than some closely related, but clearly distinct species of *Thamnophis*. These snakes were then sister to the remaining northern garter snakes. The species *T. eques* and *T. marcianus* formed a clade, as did a group of chiefly terrestrial species that included *T. brachystoma*, *T. butleri*, and *T. radix*. These species were sister to a clade including the generalist *T. elegans* and a collection of aquatic species *T. atratus*, *T. couchii*, *T. gigas*, *T. hammondii* including the terrestrial species *T. ordinoides*. Within the aquatic clade, *T. couchii* was recovered as sister to *T. hammondii* and *T. atratus* sister to *T. gigas*. The terrestrial *T. ordinoides* was sister to the clade containing *T. atratus* and *T. gigas*.

In the southern clade, there was strong congruence in the placement of a *T. cyrtopsis* and *T. pulchrilatus* clade at the base of the entire southern clade, followed by a clade of *T. fulvus* and *T. chrysocephalus* as sister to

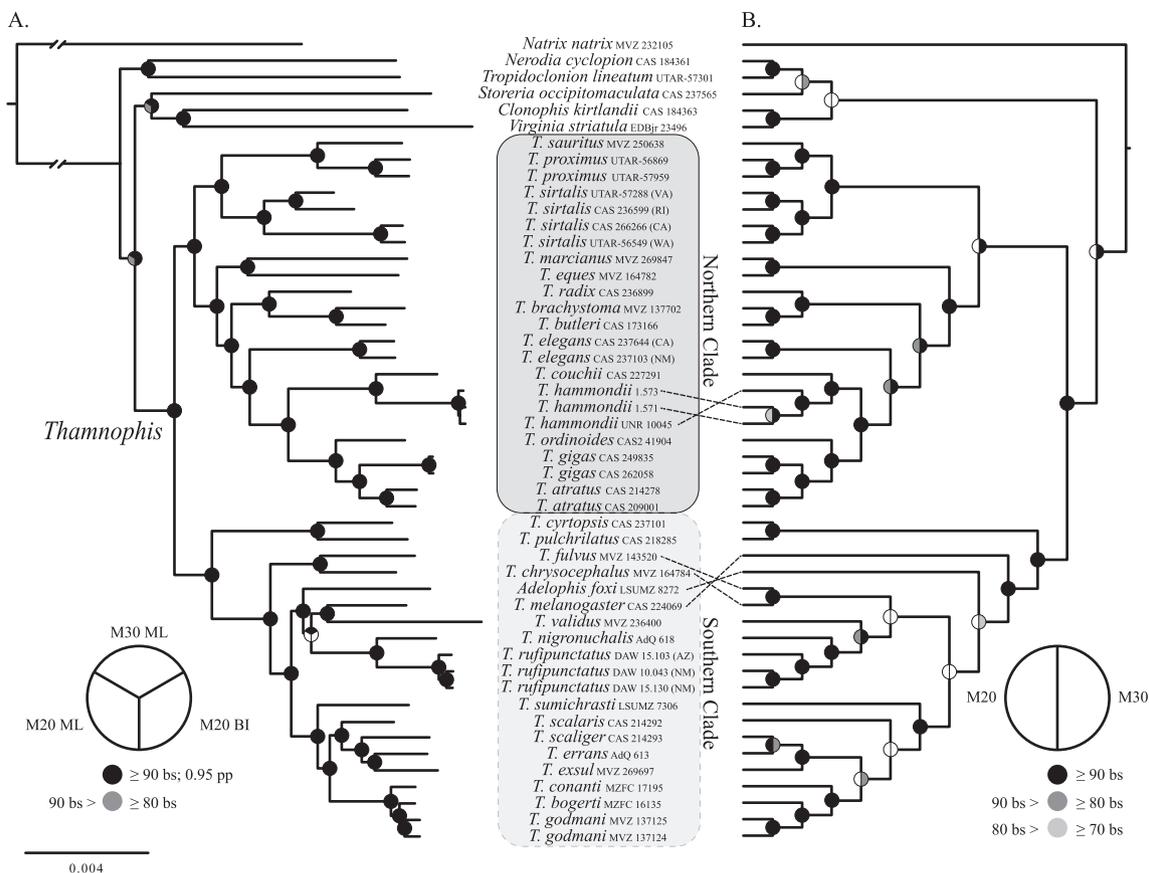


Fig. 2. Phylogenetic estimates of *Thamnophis* and allies ($n = 49$) that represent 20% (M20) and 30% (M30) missing data, with 92,244 and 198,695 parsimony informative sites, respectively. Maximum likelihood (ML) and Bayesian inference (BI) analyses of these datasets were highly congruent. (a) Topology of RAxML M30 estimate is depicted. RAxML and ExaBayes (Figs. A.1-A.2) topologies were strongly supported and recovered two clades containing largely northern (solid line, dark gray) and southern (dashed line, light gray) North American species. (b) Multispecies coalescent (MSC) model estimates from tetrad were identical for M20 and M30 datasets, and recovered congruent northern clade topology with ML and BI estimates. Symbols at nodes illustrate bootstrap (bs) and Bayesian posterior probabilities (pp) for M20 and M30 datasets.

two roughly equal sized clades containing the remaining southern species. One group formed a derived clade of Mexican species, with *T. sumichrasti* at the base, and then a clade of *T. scalaris*, *T. scaliger*, *T. errans*, and *T. exsul* and another clade of *T. conanti*, *T. bogerti*, and *T. godmani*. The second major group contained *A. foxi*, which was sister to the remaining species. This clade also held the only instance of topological conflict between M20 and M30 datasets (ML and BI), which involved the grouping of *T. melanogaster* and *T. validus* (Figs. A.1-A.2). In all ML and BI analyses *T. melanogaster* and *T. validus* were recovered as sister, as were *T. nigronuchalis* and *T. rufipunctatus*. However, the relationship among these clades in relation to *A. foxi* varied. In our M30 estimate, the clade of *T. melanogaster* and *T. validus* was sister to the clade of *T. nigronuchalis* and *T. rufipunctatus*, while our M20 ML and BI estimates recovered the *T. melanogaster* and *T. validus* clade as sister to *A. foxi*. However, the bootstrap value for this alternative arrangement was low (bs = 60).

3.3. Multispecies coalescent model estimates

The results from MSC estimates for both datasets recovered identical topologies, but sometimes with differing levels of bootstrap support (Fig. 2B; Figs. A.3). The arrangement of other natricines to each other and to garter snakes differed slightly in the MSC analyses, while *A. foxi* again rendered *Thamnophis* paraphyletic. However, our MSC estimates recovered the same split among northern and southern species as our ML and BI estimates. The relationships within the northern clade were consistent among all analyses and datasets. However, incongruencies between our MSC and other estimates were evident for some relationships within the southern clade. The placement of *T. fulvus*, *T. chrysocephalus*, *A. foxi*, and *T. melanogaster* differed, even though *T. cyrtopsis* and *T. pulchrilatus* were sister to all other members of the southern group. The placement of *T. sumichrasti* as sister to a strongly supported *T. errans*, *T. exsul*, *T. scaliger*, *T. bogerti*, *T. conanti*, and

T. godmani clade was also consistent among estimates. However, the relationships among *T. errans*, *T. exsul*, and *T. scaliger* differed between MSC and other estimates.

3.4. Divergence time estimates

Our BEAST analysis produced values of ESS > 727 across all parameters post-burnin. Divergence times and 95% height probability densities (HPD; Fig. 3; Fig. A.4) recovered TMRCA for *Thamnophis* as 12.21 Mya [95% HPD: 10.02–14.75 Mya]. The divergence time estimates for the Northern and Southern clades were 11.13 Mya [95% HPD: 9.16–13.61 Mya] and 10.54 Mya [95% HPD: 8.49–12.74 Mya], respectively.

3.5. Ancestral range reconstruction

Our ARR indicated Mexico as the most likely region for the origin of *Thamnophis* (Fig. 3; Fig. A.5). This appears to have been followed by a dispersal event north, into the southwestern United States (US), and then eastward across the US and Canada by ancestors of the northern clade. In addition, the ancestors of some species in the northern clade, such as those of *T. proximus* and *T. eques* + *T. marcianus*, appear to have then dispersed into the southern regions of North America. Diversification in the southern clade is also related to dispersal southward, with multiple postulated events across the depth of the entire lineage. Several vicariant events were also recovered across the phylogeny mainly near the tips of the southern clade and one event in the northern clade.

3.6. Ancestral state reconstruction, phylogenetic signal, and trait evolution

Our ASR of head shape recovered a fair degree of phylogenetic signal and patterning for all three traits we assessed. Relative head length and

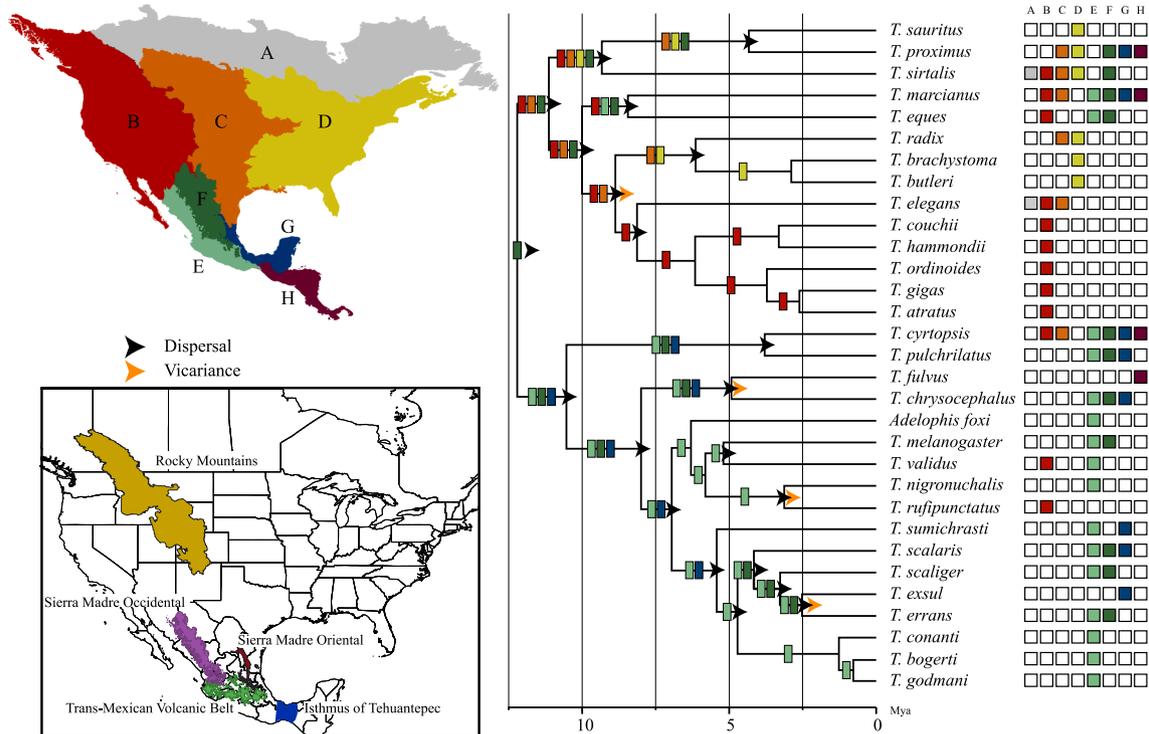


Fig. 3. Bayesian binary Markov chain Monte Carlo (BBM) ancestral range reconstruction (ARR) analysis and divergence time estimates from BEAST inferred strong biogeographic patterns and southern origin of *Thamnophis* in the mid Miocene. Ancestral ranges were calculated from summarized ecoregions across North America and ranges were constrained based on known species distributions. The map in the upper left illustrates ecoregions, and the map in the lower left illustrates major geographic features associated with vicariance events. Analysis was conducted using the ML M30 phylogenetic estimate pruned to represent a single sample per species. Each branch is labeled with the most likely ancestral range. Branch lengths depict calibrated divergence time estimates generated in BEAST.

relative muzzle width displayed stronger phylogenetic signal than relative muzzle length (Fig. 4). Relative head length showed high phylogenetic signal ($\lambda = 1.00$; $K = 1.49$; Fig. 4A); species in the southern clade had longer relative head lengths than those from the northern clade. The only exceptions were *T. eques* and *T. marcianus*, which have longer relative head lengths than other members in the northern clade. Relative muzzle width also had substantial phylogenetic signal ($\lambda = 1.00$; $K = 0.99$; Fig. 4C). However, multiple lineages appear to have evolved narrow or broad muzzles independently. The character with the lowest phylogenetic signal was relative muzzle length ($\lambda = 0.63$; $K = 0.70$), though some phylogenetic trends are clear, such as sister taxa *T. brachystoma* and *T. butleri* possessing relatively short muzzles, and sister taxa *T. atratus* and *T. gigas* sharing relatively long snouts (Fig. 4B).

Using the simulation-based approach to contrast traits with phylogenetic signal, we did not find a significant difference in the relative head length between snakes we categorized as aquatic specialists versus those we grouped as terrestrial-aquatic generalists (phylANOVA: $F_{1,29} = 1.33$, $p = 0.383$; Fig. A.6). However, we found modest differences in relative muzzle length between aquatic specialists and terrestrial-aquatic generalists (phylANOVA: $F_{1,29} = 8.57$, $p = 0.047$), and substantial differences in relative muzzle width between these groups (phylANOVA: $F_{1,29} = 26.16$, $p = 0.002$).

4. Discussion

Our phylogenomic estimates based on ddRADseq data were largely congruent across approaches and resolved chronically problematic relationships with strong support. We subsequently used this phylogenetic

hypothesis to understand the history of dispersal and vicariance events in *Thamnophis*, as well as to provide a better understanding of patterns of morphological evolution associated with feeding ecology.

4.1. Phylogeny of *Thamnophis*

Phylogenetic hypotheses for *Thamnophis* have a convoluted history, partly because early work preceded a cladistic framework and rigorous analytical methods, resulting in many taxonomic rearrangements (and disagreements) (e.g., Brown, 1904; Cope, 1892; Fitch, 1940; Fox, 1951; Ruthven, 1908). Previous phylogenetic analyses involved either allozymes (de Queiroz and Lawson, 1994; Lawson and Dessauer, 1979) or DNA sequence data from mitochondrial regions (Alfaro and Arnold, 2001; de Queiroz et al., 2002; de Queiroz and Lawson, 1994; Guo et al., 2012) or single copy nuclear genes (Guo et al., 2012; McVay et al., 2015). These analyses greatly improved our knowledge of the evolutionary relationships of garter snakes and inferences on the processes shaping lineage diversification. Nonetheless, uncertainty has remained about relationships at the base of the phylogeny, as well as conflicts at shallower evolutionary scales. In general, incongruencies across phylogenetic estimates have been suggested to be a result of insufficient power and conflicting signal in traditional markers or incomplete taxon sampling (e.g., Hallas et al., 2017; Morrison et al., 2015; Zwickl and Hillis, 2002). By expanding molecular and taxonomic sampling, our results clarify the broad topologies inferred by past studies, but also untangle previously unresolved relationships and conflict in *Thamnophis*.

Our phylogenomic analyses helped to elucidate relationships at the

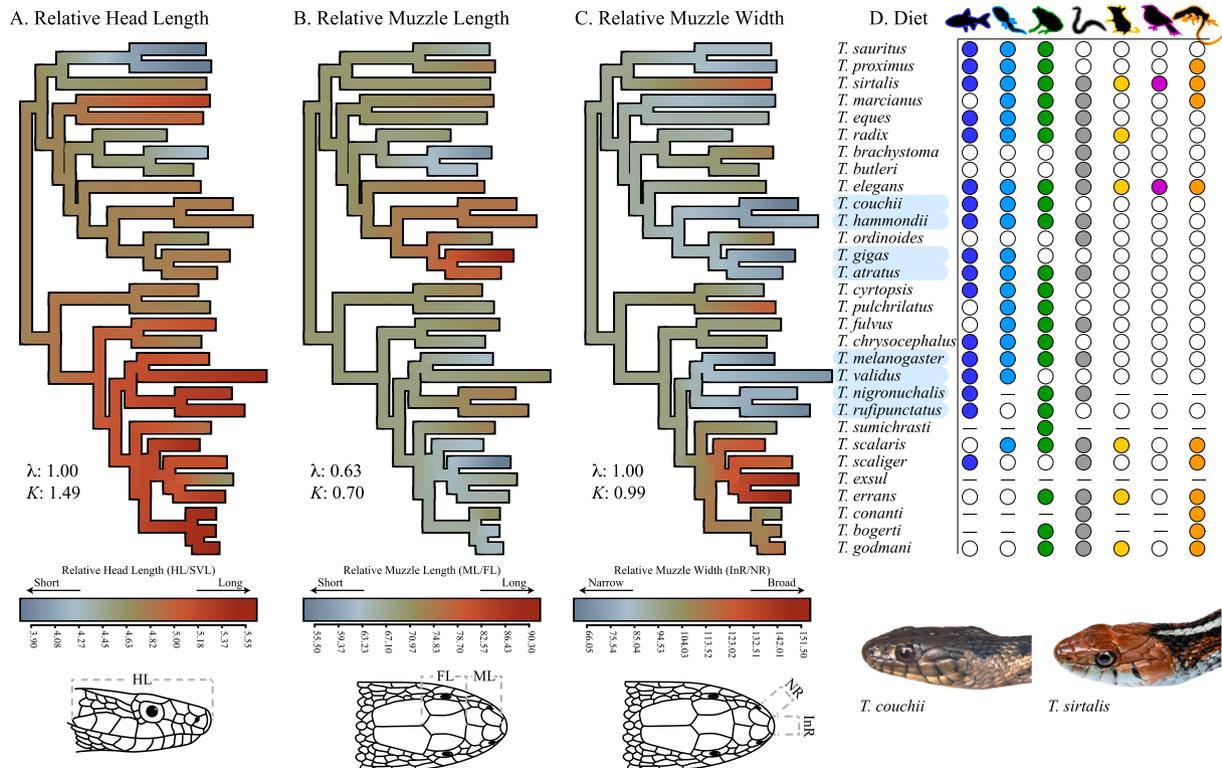


Fig. 4. Ancestral state reconstruction (ASR) and phylogenetic signal indices (Blomberg’s K and Pagel’s λ) of morphological characters associated with feeding ecology. (a) Relative head length (head length / snout-vent length), (b) relative muzzle length (muzzle length / frontal length), and (c) relative muzzle width (internasorostral contact / nasorostral contact) illustrated varying degrees of phylogenetic patterning and signal. ASR was performed using the `contmap` function in the R package `phytools` on our ML M30 phylogenetic estimate pruned to one sample per species. Taxa considered aquatic specialists are highlighted in blue. (d) Diet composition associated with each species (i.e., fish, amphibian larvae, adult amphibians, invertebrates, mammals, birds, and reptiles; see Table A.4). Colored circles indicate important components of diet, while empty circles indicate taxon not present or very rare in snake diet; dashed lines indicate insufficient information on diet. Illustration of snake head measurements (drawings by JMH). Representative aquatic specialist (*T. couchii*) with relatively long and narrow muzzle, and generalist (*T. sirtalis*) with relatively broad muzzle (photos RW Hansen). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

base of the tree. Two deep clades were often recovered in past work, with a group of mostly northern species (called either the “northern” or “widespread” clade) and a group of southern species (termed “southern” or “Mexican” clade) (de Queiroz et al., 2002; Guo et al., 2012; McVay et al., 2015). But there has been disagreement over the content of these clades, especially the southern clade, with some analyses suggesting that certain southern snakes (especially *T. chrysocephalus* and *T. fulvus*) lie at the base of the northern clade (de Queiroz et al., 2002; Guo et al., 2012). Our analyses resolve the content of these two broad clades and firmly place problematic taxa in the southern clade (e.g., *T. chrysocephalus*, *T. cyrtopsis*, *T. fulvus*, *T. pulchrilatus*), similar to McVay et al. (2015).

Prior systematic efforts also recovered a ribbon snake and common garter snake clade (*T. proximus*, *T. sauritus*, and *T. sirtalis*), but there has been substantial conflict over the placement of this group as either sister to all other garter snakes (part of the basal split) or part of a northern lineage (Alfaro and Arnold, 2001; de Queiroz et al., 2002; de Queiroz and Lawson, 1994; Guo et al., 2012). Our analyses strongly supported placement of the *T. proximus*, *T. sauritus*, and *T. sirtalis* clade as part of the northern group, sister to remaining northern clade species, a pattern also recovered in some analyses of McVay et al. (2015).

Previous work has largely focused on understanding relationships among western species of *Thamnophis* (i.e., those chiefly west of the Rockies). These taxa (*T. atratus*, *T. couchii*, *T. elegans*, *T. gigas*, *T. hammondii*, *T. ordinoides*) have been placed into a series of different species complexes (Fitch, 1940; Fox, 1951; Ruthven, 1908), which Lawson and Dessauer (1979) suggested represent individual lineages. Phylogenetic studies have generally recovered these species as closely aligned, but specific relationships among these lineages have been in conflict. For example, *T. elegans* has been recovered with lineages east of the Rocky Mountains (Alfaro and Arnold, 2001; Guo et al., 2012; McVay et al., 2015) or western garter snake species (de Queiroz et al., 2002). All of our analyses, however, recovered identical topologies that placed *T. elegans* sister to a western clade of predominantly aquatic species (Fig. 2A-B).

Lawson and Dessauer (1979) characterized a clustering of largely aquatic species represented by *T. atratus*, *T. couchii*, *T. elegans*, *T. gigas*, and *T. hammondii*. A similar grouping was recovered by both de Queiroz et al. (2002) and McVay et al. (2015), but other phylogenetic treatments have not recovered equivalent topologies (Alfaro and Arnold, 2001; de Queiroz and Lawson, 1994; Guo et al., 2012). A key issue has been the lack of clarity on the placement of *T. atratus*, which has been closely aligned to either *T. couchii* (Guo et al., 2012), *T. elegans* (de Queiroz et al., 2002), *T. gigas* (de Queiroz and Lawson, 1994; Lawson and Dessauer, 1979; Rossman and Stewart, 1987), or *T. hammondii* (McVay et al., 2015). Our results resolve relationships among these lineages and place *T. atratus* sister to *T. gigas*, and *T. couchii* sister to *T. hammondii* (Fig. 2A-B).

Lastly, we show that *A. foxi* is nested deeply within *Thamnophis* (as a member of the southern clade), consistent with all previous molecular systematic treatments of garter snakes that have included *A. foxi* (de Queiroz et al., 2002; Guo et al., 2012; McVay et al., 2015). The placement of *Adelophis* within New World natricines has always been problematic—the genus name actually means “uncertain snake” in reference to its unclear phylogenetic affinities. The two species of *Adelophis* superficially resemble other semi-fossil natricines, such as *Storeria* and *Tropidoclonion*, and have even been placed in these other genera at times (reviewed in Rossman and Wallach, 1987). They possess unique supralabial and infralabial scale counts and specialized osteological traits of the skull (Rossman and Blaney, 1968; Rossman and Wallach, 1987). However, the phylogenetic utility of the unusual facial scale counts has been called into question as uninformative autapomorphies (de Queiroz et al., 2002), and the distinct features of the skull are thought to be derived adaptations for a semi-fossil lifestyle (Rossman and Blaney, 1968). Furthermore, *Adelophis* share an undivided anal plate with *Thamnophis* (Conant, 1961), a scale feature that is considered a useful synapomorphy for garter snakes (de Queiroz et al., 2002). Because the

traits that make *Adelophis* distinct appear to reflect ecological divergence and not evolutionary history, and because of the consistent placement of *Adelophis* within *Thamnophis* across independent datasets (mtDNA, nDNA, ddRADseq) and analyses (MP, ML, BI, MSC), we formally recommend subsuming the genus *Adelophis* into *Thamnophis*. Some might refrain from formal changes to *A. copei* until that taxon can be suitably evaluated in a phylogenetic analysis. However, the sister relationship between *A. foxi* and *A. copei* has never been challenged (Rossman and Blaney, 1968; Rossman and Wallach, 1987), and it seems highly unlikely that *A. copei* would sit outside of *Thamnophis* while *A. foxi* has roots deep in the southern clade of *Thamnophis*. Thus, a taxonomic revision is well overdue, and we propose discarding the genus *Adelophis* and placing both *A. foxi* and *A. copei* into *Thamnophis*.

4.2. Biogeographic history

A Mexican origin of *Thamnophis*, first proposed by Ruthven (1908), has been broadly supported by subsequent phylogenetic studies (Alfaro and Arnold, 2001; de Queiroz et al., 2002; de Queiroz and Lawson, 1994). Our analysis further bolsters this hypothesis (Fig. 3), and infers a mid-late Miocene origin for the genus (Fig. 3, Figs. A.4-A.5). This result is further supported by similar estimates of mid Miocene (McVay et al., 2015) and late Miocene (Guo et al., 2012) origins of the group. Within *Thamnophis*, geography and historical events have clearly influenced the diversification and distribution of species, as illustrated by our ARR, which recovers multiple dispersal and vicariance events across the phylogeny. As noted above, we find a strong biogeographic division between the northern and southern garter snake clades. The species of the northern clade are found almost entirely in northern North America with few a few exceptions. The most likely ancestral range of the clade appears to be in western North America with dispersal events eastward across the Great Plains and into eastern North America. A few species in the northern clade also showed dispersal events into the southern regions of North America. These involved *T. proximus* in addition to *T. eques* and *T. marcianus* whose distributions include regions of the MTZ.

The major vicariant event recovered within the northern clade is between a clade of largely western endemics (*T. atratus*, *T. couchii*, *T. elegans*, *T. gigas*, *T. hammondii*, and *T. ordinoides*) and a clade of northeastern and midwestern snakes (*T. brachystoma*, *T. butleri*, and *T. radix*). This event may be explained by processes associated with the Rocky Mountains (Fig. 3). Even though uplift of the range far precedes these lineages, orogenesis throughout the west (Brown, 1997; Potts and Behrensmeyer, 1992) and the aridification of the Great Plains during the Miocene-Pliocene transition (Axelrod, 1985) might have caused the initial split. Pook et al. (2000) suggested this Miocene-Pliocene climatic shift east of the Rocky Mountains was also responsible for the deepest division in the *Crotalus viridis* complex.

The species of the southern clade are found principally in Mexico and neighboring countries to the south, and the ancestral range mainly represents the MTZ. Our analyses suggest multiple dispersal events across Mexico, such as in the ancestor of *T. cyrtopsis* and *T. pulchrilatus*, but also northward, such as the northwest dispersal of *T. validus* into Baja California (de Queiroz and Lawson 2008). Multiple vicariant events also appear to have influenced cladogenesis in the southern clade. In fact, these events seem associated with the geographic features of the MTZ (e.g., Isthmus of Tehuantepec, Sierra Madre Oriental, Sierra Madre Occidental, Trans-Mexican Volcanic Belt) thought to have similarly shaped the histories of other lineages (e.g., Bryson et al., 2013; Corona et al., 2007; Daza et al., 2010; Marshall and Liebherr, 2000; Mastretta-Yanes et al., 2015; Morrone, 2010). For example, the Isthmus of Tehuantepec is a biogeographic barrier associated with genetic structure and allopatric speciation in a broad range of taxa (Castoe et al., 2009; Daza et al., 2010; León-Paniagua et al., 2007; Mulcahy et al., 2006; O’Connell et al., 2017; Venkatraman et al., 2019). This narrow strip of lowland in southern Mexico divides surrounding highlands and

separates montane flora and fauna by a low expanse of savanna with a distinctive climate (Marshall and Liebherr, 2000). Multiple tectonic episodes since the late Miocene (6 Mya) formed the Isthmus of Tehuantepec (Barrier et al., 1998), thereby segregating formerly connected species and creating an ecological filter (Marshall and Liebherr, 2000; Mulcahy et al., 2006). The high elevation montane sister taxa *T. chrysocephalus* and *T. fulvus* are bifurcated by the Isthmus of Tehuantepec, and our divergence time estimates coincide with the formation of the Isthmus, likely responsible for the isolation and subsequent allopatric evolution of these two species (de Queiroz et al., 2002).

Complex patterns of vicariance and dispersal also seem responsible for diversification in southern clade garter snakes found in montane habitats of the major mountain chains of Mexico. An assemblage of *Thamnophis* (*T. bogerti*, *T. conanti*, *T. errans*, *T. exsul*, *T. godmani*, *T. scalaris*, *T. scaliger*, *T. sumichrasti*), referred to as the “Mexican highland” clade (de Queiroz et al., 2002), occupy high elevation pine-oak and tropical deciduous forests of the Sierra Madre Occidental, Sierra Madre Oriental, Trans-Mexican Volcanic Belt (TMVB), and Sierra Madre del Sur (Rossman et al., 1996; Rossman and Burbrink, 2005). The north–south running Sierra Madre Occidental and Sierra Madre Oriental are separated by the vast Mexican Plateau of arid desert scrub and grassland, forming an inhospitable barrier for the Mexican highland species. However, these two ranges are connected along their southern ends by the TMVB, which contains suitable habitat to permit some biotic connection between the two ranges (Corona et al., 2007; Morrone, 2010). Indeed, populations of a number of vertebrates of the Sierra Madre Occidental and Sierra Madre Oriental exhibit close genetic relatedness despite being isolated on these ancient ranges by hundreds of kilometers, such as the jay *Aphelocoma ultramarina*, (McCormack et al., 2008) and spiny lizard *Sceloporus scalaris* (Bryson et al., 2012). We recovered a similar pattern with the sister relationship of *T. errans* and *T. exsul*, which persist on the Sierra Madre Occidental and Sierra Madre Oriental, respectively. It is possible that dispersal across the Mexican Plateau, through pine-oak corridors that developed during the last glacial maximum, could explain the tight relationships between species in these two ranges, as proposed for several other taxa (e.g., Bryson et al., 2011a,b; Martin, 1958; Van Devender, 1990). However, our divergence time estimates instead favor the TMVB as the probable link between the Sierra Madre Occidental and Sierra Madre Oriental. The uplift of the TMVB, which occurred in multiple episodes during the mid to late Miocene (reviewed in Ferrari et al., 2012; Gómez-Tuena et al., 2007) coincides well with diversification of the Mexican highland clade (6–2 Mya; Fig. 2). This uplift could have allowed connection and entry into the Sierra Madre Occidental and Sierra Madre Oriental by highland *Thamnophis*. Furthermore, these multiple volcanic episodes could have fragmented the ancestors of some of the Mexican highland species (such as TMVB species *T. scalaris* and *T. scaliger*), as seen in the diversification of other montane species (e.g., Blair and Sánchez-Ramírez, 2016; Bryson et al., 2012; Bryson et al., 2011a, 2011b; Mastretta-Yanes et al., 2015; Rocha-Méndez et al., 2019; Ruiz-Sanchez and Specht, 2013). Because of the large distributions of some of these species, some of which are also highly fragmented, focused phylogeographic and population genetic studies are needed to untangle the potentially complex biogeographic history of Mexican highland *Thamnophis*.

4.3. Morphological evolution and feeding ecology

Diet, foraging mode, and habitat type have been proposed as potential drivers of head shape evolution in snakes because the cranium is the major mechanism by which these limbless predators acquire and ingest food (Greene, 1997; Hampton, 2011). Patterns of head morphologies associated with environment and feeding ecology have been observed in across multiple snake lineages, including homalopsids (Fabre et al., 2016), pythonids and boids (Esquerré and Keogh, 2016) and xenodontines (Klaczko et al., 2016). Garter snakes exhibit extensive variation in diet across the phylogeny (Fig. 4; Table A.4), and employ

many modes of foraging and feeding behaviors that appear to be suited for environment and prey type (de Queiroz, 2003; Drummond, 1983; Halloy and Burghardt, 1990; Schaeffel and de Queiroz, 1990; Welsh and Lind, 2000). Thus, we expect that aspects of head shape will reflect not just phylogenetic history, but also feeding ecology (e.g., Segall et al., 2016). Specifically, species that mainly hunt aquatic prey are expected to have relatively long and narrow muzzles to reduce hydrodynamic drag, while those with broader or mostly terrestrial diets should have wider muzzles and deeper heads to accommodate an array of potentially large prey (Fabre et al., 2016; Hampton, 2011; Herrel et al., 2008; Savitzky, 1983). Given these eco-morphological associations, we mapped cranial variation onto the phylogeny to first determine the phylogenetic component of trait variation, and then tested whether head shape differs between species that are aquatic specialists versus those that are terrestrial-generalists.

All three head shape traits (relative head length, relative muzzle length, relative muzzle width) displayed moderate to strong phylogenetic signal (Fig. 4). However, morphological variation, especially in muzzle width, also differed between snakes with distinct foraging modes (i.e., between aquatic specialists and others). Relative head length displayed the strongest phylogenetic signal, suggesting a high degree of phylogenetic conservatism. This trait contained little variation across almost the entire southern clade—nearly all possess similarly long heads relative to the body. Interestingly, many of these species seem to take highly terrestrial prey, such as mice, lizards, and plethodontid salamanders (Fig. 4; Table A.4). Nevertheless, better natural history and diet data are needed for many of these species to determine if this aspect of the skull is invariant due to shared feeding ecologies, or if there are constraints on head shape despite differences in prey, feeding mode, habitat use, or other factors.

Relative muzzle length and width, both of which have been suggested to correlate with aquatic specialization (Fabre et al., 2016; Hampton, 2011; Herrel et al., 2008; Savitzky, 1983), showed greater variation across the tree. In fact, our ASR analyses suggest the repeated evolution of relatively narrow muzzles from ancestors with intermediate muzzle widths. This is evident in our two groups of aquatic specialists, which evolved twice in *Thamnophis*: once in a group of western endemics of the northern clade (*T. atratus*, *T. couchii*, *T. gigas*, *T. hammondii*) and also in the sister group of the Mexican highland species of the southern clade (*T. melanogaster*, *T. nigronuchalis*, *T. rufipunctatus*, and *T. validus*). All of these species are highly aquatic and forage for fully aquatic or semi-aquatic prey in the water using derived behaviors (de Queiroz, 2003; Drummond, 1985, 1983; Ernst and Ernst, 2003; Rossman et al., 1996; Stebbins, 2003; Tanner, 1985; Fig. 4; Table A.4). Our phyANOVA suggested these species have significantly longer and narrower muzzles compared to all other garter snakes, despite phylogenetic signal in these traits. Thus, the independent evolution of aquatic specialization in these groups appears to have led to convergent morphological change, a pattern seen across natricine snakes (Fabre et al., 2016; Herrel et al., 2008; Hibbitts and Fitzgerald, 2005; Segall et al., 2019; Vincent et al., 2009; Young, 1991). Interestingly, *T. ordinoides*, which feeds primarily on terrestrial and fossorial invertebrates and contains a broader and shorter muzzle, is nested within the western aquatic specialists, demonstrating the evolutionary lability of feeding mode, diet, and morphology in *Thamnophis*. Likewise, members of the Mexican highland clade (*T. errans*, *T. exsul*, *T. scalaris*, and *T. scaliger*) have evolved relatively short broad muzzles that may impart a mechanical advantage when consuming struggling prey (such as lizards) or permit a more generalist diet (Hampton, 2011).

Our analyses indicated interesting morphological trends across *Thamnophis*, including patterns of convergent evolution. However, these efforts are admittedly broad-brushed, and a more complete understanding of morphology associated with feeding ecology (e.g., behavioral and natural history) will require more refined work on multivariate traits (e.g., Fabre et al., 2016; Herrel et al., 2008; Hampton, 2011). For example, how do geographic variation and ontogenetic shifts in diet

contribute to patterns of morphological variation? As an example, the diet of *T. validus* differs between Baja California and mainland Mexico populations, and those from mainland Mexico shift from amphibians at an early age to fish as adults (de Queiroz et al., 2001). In addition, differences in habitat use (e.g., lentic or lotic systems), foraging mode, or behavioral tactics (e.g., frontal or lateral aquatic feeding attacks) may influence trait evolution (Alfaro, 2002; Drummond, 1983; Herrel et al., 2008; Hibbitts and Fitzgerald, 2005). Clearly, additional work is needed to more fully understand the interplay between diet, ecology, behavior and morphological evolution in *Thamnophis*.

5. Conclusion

Here, we leveraged genome-wide high-throughput sequencing data and near complete taxon sampling to estimate a robust phylogeny for *Thamnophis*. Our results strengthen several previous phylogenetic conclusions while also resolving a number of historically problematic relationships (Alfaro and Arnold, 2001; de Queiroz et al., 2002; Guo et al., 2012; McVay et al., 2015). Our analysis of spatiotemporal patterns of diversification bolstered the hypothesis of a Mexican origin of the genus (Alfaro and Arnold, 2001; de Queiroz et al., 2002; de Queiroz and Lawson, 1994; McVay et al., 2015; Ruthven, 1908), likely in the mid-Miocene (Guo et al., 2012; McVay et al., 2015). Our analyses also suggest a complex history of dispersal and vicariance that warrants more detailed study within specific clades. Lastly, we examined patterns of morphological evolution thought to be associated with feeding ecology. All three cranial traits displayed some degree of phylogenetic signal, yet selection appears to have nonetheless influenced head shape in relation to foraging strategy. In fact, *Thamnophis* display some degree of convergent evolution associated with aquatic specialization. More generally, our phylogeny provides a useful evolutionary hypothesis for a range of future ecological and evolutionary investigations within *Thamnophis*.

CRedit authorship contribution statement

Joshua M. Hallas: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing, Visualization. **Thomas L. Parchman:** Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing, Visualization. **Chris R. Feldman:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing, Visualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary materials

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ympcv.2021.107374>.

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