



Testing monophyly without well-supported gene trees: Evidence from multi-locus nuclear data conflicts with existing taxonomy in the snake tribe Thamnophiini



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ABSTRACT

Ideally, existing taxonomy would be consistent with phylogenetic estimates derived from rigorously analyzed data using appropriate methods. We present a multi-locus molecular analysis of the relationships among nine genera in the North American snake tribe Thamnophiini in order to test the monophyly of the crayfish snakes (genus *Regina*) and the earth snakes (genus *Virginia*). Sequence data from seven genes were analyzed to assess relationships among representatives of the nine genera by performing multi-locus phylogeny and species tree estimations, and we performed constraint-based tests of monophyly of classic taxonomic designations on a gene-by-gene basis. Estimates of concatenated phylogenies demonstrate that neither genera are monophyletic, and this inference is supported by a species tree estimate, though the latter is less robust. These taxonomic findings were supported using gene tree constraint tests and Bayes Factors, where we rejected the monophyly of both the crayfish snakes (genus *Regina*) and the earth snakes (genus *Virginia*); this method represents a potentially useful tool for taxonomists and phylogeneticists when available data is less than ideal.

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1. Introduction

Accurate estimates of phylogeny from molecular data offer vital information for understanding the evolution of any clade of organisms. One such group is the Natricine snakes; these snakes occur on all continents but Antarctica and South America, and are represented in the New World by approximately 60 species (the entire tribe Thamnophiini). Natricine snakes are a particularly compelling focus for phylogenetic analysis because representatives of this group occupy broad ecological niche space, from complete terrestrial to almost exclusive aquatic habitat, and from broad generalists to stenophagic diets (Gibbons and Dorcas, 2004; Rossman et al., 1996). Data from molecular phylogenies enable us to understand the evolutionary lability of traits related to habitat and diet, and to estimate how quickly these traits evolve (Alfaro, 2003; King et al., 2009; Rossman et al., 1996; Schaeffel and de Queiroz, 1990; Wusterbarth et al., 2010).

Members of the tribe Thamnophiini are particularly flexible in diet, with members feeding on fish, amphibians, reptiles, mammals, annelids, insects, mollusks, and crustaceans (Gibbons and Dorcas, 2004; Rossman et al., 1996). One feeding specialty highlights the important role played by molecular phylogenies: the four

crayfish specialists in the genus *Regina*. There is debate among scholars as to whether these snakes represent a single or two monophyletic groups. While ecology and feeding behavior suggest a shared ancestry, other characters such as dental morphology (Rossman, 1963), and scale microtexture (Price, 1983) have led some scholars to place two of these species, the Glossy (*R. rigida*) and Striped Crayfish Snakes (*R. alleni*) into their own previously described genus, *Liodytes* (Cope, 1885); but see Rossman (1963, 1985). Most recently, Alfaro and Arnold (2001) suggested that *Regina* is not monophyletic, based on phylogenetic analysis of mitochondrial sequence data. Interestingly, one well-supported clade from this study contained the two “*Liodytes*” species, and nested within, the Swamp Snake (*Seminatrix pygaea*), which lacks many of the derived morphological characteristics present in the other two species (Dowling, 1950; Price, 1982). If this phylogeny is accurate, the feeding specializations associated with crayfish eating are either convergent or have been lost in some members. However, our ability to make this inference depends on our ability to estimate the phylogeny from the data; in this case the most recent estimate was produced from three mitochondrial genes (ND2, CYTB, 12S) for 27 ingroup species representing eight genera (Alfaro and Arnold, 2001).

The Thamnophiini also contain the earth snakes (genus *Virginia*), represented by two species (*V. striatula* and *V. valeriae*), another problematic group. Originally placed in the novel genera

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(*Haldea striatula* and *V. valeriae*) by Baird and Girard (1853), the former was submerged within the *Virginia* by Garman (1883). There have since been a number of studies supporting or rejecting this move (for a review, see Rossman and Wallach (1991)), including allozyme data that lends support to original designation (Lawson, 1985), however no taxonomic changes have been formally accepted. To date DNA sequence data has only been published for one species, leaving this taxonomic change untested in a modern phylogenetic framework.

Here we use newly-collected molecular sequence data to address the taxonomic status of the two natricine snake genera *Regina* and *Virginia*, the latter of which contains two (or three) species, but to date has only been represented by one species in molecular genetic studies. Specifically, we ask “Is *Regina* a monophyletic genus, or does it represent two or more independently evolving lineages?” and “Are the earth snakes (genus *Virginia*) sister taxa?” We will address these questions with a multi-locus, mitochondrial and nuclear dataset containing one or more representatives of all putative genera in *Thamnophiini*.

2. Materials and methods

2.1. Sample preparation and sequencing

Tissue samples of specimens were obtained from the Louisiana State University Museum of Natural Science (Supplemental Table S1). We extracted total DNA from tissues following a modified version of the protocol described by Aljanabi and Martinez (1997), where tissues were initially digested overnight in 300 μ L of Puregene[®] Cell Lysis Solution (QIAGEN catalog no. 158906) and 2.5 μ L Proteinase K (New England Biolabs no. P8102S) prior to following the standard protocol. DNA samples were then quantified via Nanodrop (Thermo Scientific, Waltham, MA) and diluted to a final concentration of 10–25 ng/ μ L.

Polymerase chain reactions were performed for each individual for five nuclear and two mitochondrial genes (Table 1). Reactions were performed in 25 μ L with the following reagent concentrations: 0.4–1 ng/ μ L tDNA, 0.4 μ M each primer, 0.2 μ M dNTPs, 1 \times Standard Taq reaction buffer (New England Biolabs) and 0.5 units of Taq DNA polymerase (New England Biolabs no. M0267). For all but ND4 (55 $^{\circ}$ C annealing temperature), thermocycling was performed with an initial melting step of 2 min at 95 $^{\circ}$ C 30 cycles of: 30 s at 95 $^{\circ}$ C, 15 s at 50 $^{\circ}$ C and 30 s at 72 $^{\circ}$ C, followed by 10 min at 72 $^{\circ}$ C. Sequence analysis was performed on an ABI 3130XL (Applied Biosystems, Foster City, CA) after sequencing was performed using BigDye v 3.1, following manufacturers

Table 1
Primers and sources of gene fragments used in this study. Shown for each gene are the primer sequence (in 5' to 3' orientation) and the source of each primer.

Gene	Oligo (5'–3')	Reference
BDNF	F GACCATCCTTTCTKACTATGTTATTTCATACCTT	Leache and McGuire (2006)
	R CTATCTCCCTTTTAAATGGTCAGTGTACAAAC	
FSHR	F CCDGATGCCTTCAACCCVGTGA	Wiens et al. (2008)
	R CCRAAYTTRCTYAGYARRATGA	
CYTB	F TGATCTGAAAAACACCGTGTGA	Alfaro and Arnold (2001)
	R AATGGGATTTTGCAATGTCTGA	
MC1R	F TCAGCAACCTGGTGGA	Austin et al. (2010)
	R ATGAGGTAGAGGCTGAAGTA	
ND4	F TGACTACAAAAGCTCATGTAGAAGC	Forstner et al. (1995)
	R TTTTACTTGGATTGCACCA	
NT3	F ATGTCCATCTTGTATGTATATTT	Wiens et al. (2008)
	R ACRAAGTTTGTGTCTGAAGTC	
R35	F TCTAAGTGTGGATGATYTGAT	Fry et al. (2006)
	R CATCATTGGRAGCAAAAGAA	

instructions. Both PCR and cycle sequencing products were purified following an ethanol precipitation procedure. Sequences were edited using Sequencher 4.8 (Genecodes, Ann Arbor, MI) and aligned using Muscle (Edgar, 2004) and manually verified. Phasing of ambiguous alleles was performed using PHASE 1.4 (Stephens et al., 2001); data was formatted using SEQPHASE (Flot, 2010). See below for treatment of sites that could not be resolved with greater than 90% confidence using PHASE.

2.2. Gene tree estimation

Phylogenetic estimates were produced for each nuclear gene fragment and the combined mitochondrial fragments using MrBayes 3.2.1 (Ronquist et al., 2012). We chose models of sequence evolution following results from DT-ModSel (Minin et al., 2003). For each gene, a four-chain (three cold, one hot) Markov Chain Monte Carlo (mcmc) was run for 5,000,000 generations, sampling every 500, or until standard deviations of split frequencies fell below 0.01, ensuring proper mixing of chains. A burn-in of 25% of sampled steps (program default) was used for all genes; support for nodes was assessed using Bayesian posterior probability (BPP) values.

An important assumption of the coalescent model is that genes are evolving in a neutral fashion. Violation of this assumption may lead to branch length heterogeneity among gene trees (Edwards, 2009) and instances of strong directional selection may lead to topological bias in the estimated gene tree. After determining reading direction and frame for each gene, we tested for evidence of purifying selection ($dS > dN$) by using the codon-based z-test of selection in Mega5 (Tamura et al., 2011), implementing the “overall average” function.

2.3. Phylogeny estimation

Because any recombinant unit of DNA (such as the mitochondrial genome) is subject to the stochastic process of gene coalescence, its genealogy may not reflect the actual pattern of species divergence (Degnan and Rosenberg, 2009). Thus, any phylogenetic estimate based on sequence from a single recombining unit may be biased in both branch length and topology, and assuming a single underlying genealogy for multiple, unlinked genes (as is the case when data are concatenated) may lead to biased or even positively misleading estimates (Degnan and Rosenberg, 2006) of the containing phylogeny.

Estimates of phylogeny under both the concatenated and multi-species coalescent model were produced using BEAST 1.7.1 (Drummond et al., 2012). Substitution models obtained from DT-ModSel were implemented for each gene, substitution rates and clocks were unlinked across genes, and clock model for each gene was set to uncorrelated relaxed – lognormal, with uniform prior with a range of 0–10. The MCMC was run for 100,000,000 generations, sampling every 10,000 generations. For the multi-species coalescent, topologies were unlinked among genes (mitochondrial topologies remained linked) and the *BEAST prior was implemented. Each analysis was performed twice, and posterior distributions of parameters were compared to ensure consistency across runs using Tracer 1.5 (Rambaut and Drummond, 2009).

2.4. Phasing

Coalescent-based species tree estimators rely on population genetic parameters and processes to estimate relationships among populations. Parameters like $\theta = 4N_e\mu$ can be better estimated given more information about the allele frequencies within populations. When more than one site is ambiguous within an individual sequence, determining the alleles (experimentally or

via estimation) representing this sequence is important for estimating coalescent parameters, and can reveal anomalous shared ancestry of alleles among populations. As the populations being studied become more distantly related, the probability of shared alleles becomes lower, leading to the idea that phasing of ambiguous data will be less important to estimation of gene trees and the containing species tree. Thus, in a case such as ours, where divergence between the species included in the investigation are likely greater than the expected time to coalescence of all alleles within a given species, ambiguous sites that cannot be phased may represent allelic autapomorphies that will not affect the outcome of the analysis. To test whether this is the case, we used Paup* (Swoford, 2003) to build neighbor-joining trees containing all possible phases for each gene with ambiguous data, with the null expectation that all possible alleles should form a monophyletic group. Depending on the depth of relationships being investigated, violation of this expectation may be attributed to one or more causes, including incomplete or anomalous lineage sorting, introgression, gene duplication and loss, and selection.

2.5. Tests of monophyly

Monophyly of a previously designated or putative taxonomic group may be rejected when there is statistical support based on BPP for a branch or branches within trees that contain topologies incongruent with the taxonomic hypothesis. However, when monophyly is not supported by the phylogenetic estimate, but there is not enough statistical support (i.e. BPP > 0.95) to reject monophyly, a comparison of marginal likelihood estimates between two models, one topologically constrained to include the monophyletic clade to be tested, and one topologically unconstrained, can be used to evaluate monophyly. Here we test a number of putative monophyletic groups within *Thamnophiini*: (1) *Regina* (Rossman, 1963), (2) *R. septemvittata* and *R. grahamii* (to the exclusion of the *Liodytes* group; Lawson (1985)), (3) *Liodytes* (Price, 1983), (4) *Liodytes* and *Seminatrix* (Alfaro and Arnold, 2001), (5) *Virginia* (Garman, 1883) and (6) *Storeria* (Baird and Girard, 1853) as a genus previously supported by both molecular and morphological data (Trapido, 1944; Alfaro and Arnold, 2001). We constrained the topology for each of the above groups and performed a stepping stone run of 10,200,000 generations (50 steps with stationarity being reached in each step) from which we obtained a marginal likelihood estimate; each of these estimates were compared using Bayes Factors (Kass and Raftery, 1995) to the marginal likelihood estimate obtained from an unconstrained MrBayes run of the same length. The stepping stone function (Xie et al., 2011) implemented in MrBayes 3.2, offers an improved estimation of marginal likelihood over harmonic mean estimation. In addition to these tests, we measure support for each of the above groupings by observing both BPP > 0.95 that support or exclude monophyly; we additionally filtered and counted trees containing these groups for each gene tree and species tree posterior distributions using the constraint filter commands in PAUP*.

3. Results

3.1. Gene trees

A variation of the two-substitution site model was chosen for each gene (Supplemental Table S2). Within each gene, topologies of consensus trees (maximum clade credibility) were consistent between runs. Support (BPP) was generally low among all nuclear loci, but high for many nodes within the mitochondrial gene tree estimate (Fig. S1). Topologies were inconsistent among genes; however no strongly supported discordance between topologies

was present. The codon-based z-test of selection strongly rejected ($p < 0.01$) neutrality in favor of purifying selection across all genes tested (Supplemental Table S2).

3.2. Phylogeny estimation

Phylogenies were estimated under two evolutionary models: concatenation and a coalescent-based species tree approach (Fig. 1). Topologies were incongruent at multiple nodes across the tree, however posterior probabilities were low for discordant nodes. Both estimates split the ingroup into a clade consisting of mostly fossorial snakes (*Clonophis*, *Regina alleni* and *rigida*, *Seminatrix*, *Storeria* and *Virginia*) and a mostly semiaquatic and terrestrial group (*Adelophis*, *Nerodia*, *Regina grahamii* and *septemvittata*, *Thamnophis*, and *Tropidoclonion*). Neither estimate recovered *Regina* or *Virginia* as monophyletic, and the concatenated estimated rejected these groupings with greater than or equal to 0.95 Bayesian posterior probability.

3.3. Phasing

We used PHASE to estimate alleles for each nuclear gene; in all but two (MC1R, NTF3), alleles could be resolved with high confidence (Supplemental Fig. S2). For NTF3, all possible phases for each species coalesced prior to the nearest interspecific node (i.e., the possible alleles were monophyletic). For MC1R, alleles representing *Adelophis foxi* were not monophyletic: two possible phase resolutions yielded similar results (Supplemental Fig. S2). These heterozygous sites were excluded from analyses.

3.4. Tests of monophyly

We compared the marginal likelihood estimates of a topologically constrained and unconstrained run of MrBayes for five possible monophyletic groups within *Thamnophiini* (Table 2). Results strongly suggest that the classic taxonomic groupings of the crayfish snakes and the earth snakes are not valid, and there was also evidence against a monophyletic group containing *Regina grahamii* and *R. septemvittata*. The only strong positive evidence is shown for the group containing *R. alleni* and *R. rigida* (“*Liodytes*”), along with *Seminatrix pygaea*, with mixed evidence for grouping the two “*Liodytes*” as sister. The genus *Storeria* was supported as monophyletic by four of six genes, with two others neither supporting nor rejecting this relationship.

4. Discussion

Taxonomists have traditionally employed morphological, ecological and distributional measures to diagnose taxa, and infer relationships among them. In the last several decades, molecular sequence data has played an increasing role in this field, and advances in methods of data acquisition and analysis have led to changes in the way species are discovered and diagnosed (Wiens, 2007). However, methods for estimating phylogenies from these data have continued to evolve. It has been argued that the field of molecular systematics has been subject to a paradigm shift (Edwards, 2009) related to how multilocus data are analyzed. Since we seek to recover the pattern of diversification across species, rather than to estimate a genealogy of a particular gene with the hopes that this genealogy reflects the underlying species tree, we favor species tree approaches that model the divergence of evolutionary lineages. We agree with Edwards (2009) that concatenation is not appropriate for the data collected here. However, we have also uncovered evidence that the data collected here are subject to purifying selection, and these results demonstrate that our

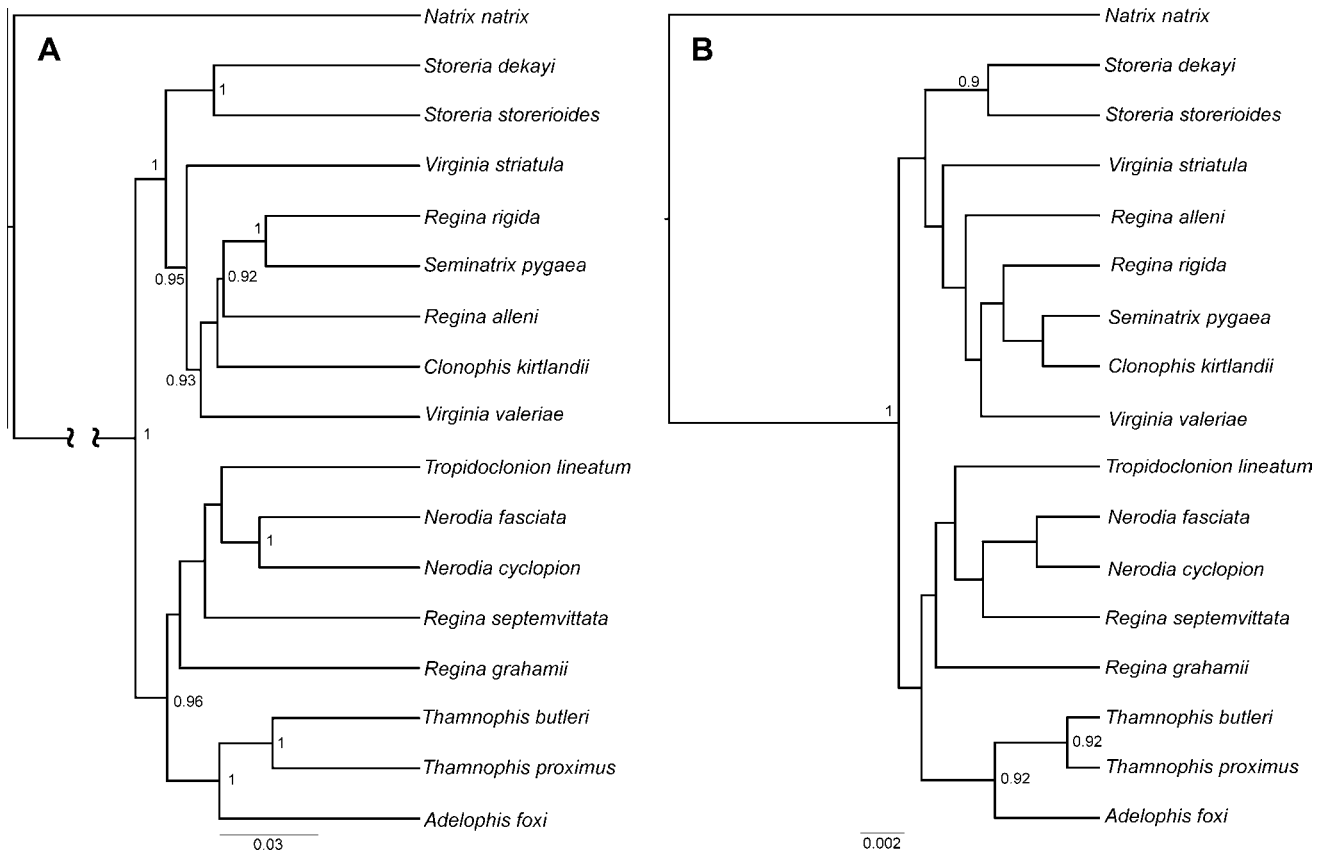


Fig. 1. Multi-locus Bayesian maximum clade credibility estimates. (A) Concatenated phylogeny; (B) multi-species coalescent. Unlabeled nodes were not supported with greater than 0.9 Bayesian posterior probability.

Table 2
Bayes factors of stepping-stone-based estimates of marginal likelihood for five putative and one well supported monophyletic groupings. Strong favor (–5 to –3), substantial favor (–1.5 to –3), substantial rejection (1.5–3), strong rejection (3–5), very strong rejection (5–6.6), decisive rejection (>6.6).

Taxonomic constraint	Gene						
	BDNF	FSHR	MC1R	MT	NT3	R35	
<i>“Regina”</i>							
+	–1082.39	–1057.59	–1013.34	–6399.34	–1441.52	–1454.82	
–	–1079.03	–1054.5	–1003.93	–6338.73	–1432.61	–1441.88	
BF	3.36	3.09	9.41	60.61	8.91	12.94	
<i>“Virginia”</i>							
+	–1079.22	–1058.33	–1002.63	–6341.72	–1434.54	–1445.69	
–	–1079.21	–1054.47	–1003.91	–6338.55	–1432.6	–1441.86	
BF	0.01	3.86	–1.28	3.17	1.94	3.83	
<i>“Liodytes”</i>							
+	–1078.65	–1053.27	–1009.79	–6347.99	–1430.45	–1440.63	
–	–1079.09	–1054.6	–1003.91	–6338.76	–1432.84	–1442.23	
BF	–0.44	–1.33	5.88	9.23	–2.39	–1.6	
<i>Liodytes + Seminatrix</i>							
+	–1078.53	–1051.81	–1014.88	–6336.13	–1429.03	–1439.02	
–	–1079.07	–1054.31	–1003.93	–6338.63	–1432.56	–1442.16	
BF	–0.54	–2.5	10.95	–2.5	–3.53	–3.14	
<i>R. grahamii + R. septemvittata</i>							
+	–1083.1	–1059.13	–1005.02	–6345.86	–1431.75	–1448.41	
–	–1078.97	–1054.34	–1003.95	–6338.75	–1432.58	–1441.85	
BF	4.13	4.79	1.07	7.11	–0.83	6.56	
<i>Storeria</i>							
+	–1072.15	–1055.82	–1000.97	–6335.41	–1427.72	–1440.19	
–	–1073.37	–1054.46	–1005.26	–6343.94	–1429.57	–1442.27	
BF	–1.22	1.36	–4.29	–8.53	–1.85	–2.08	

data violate one assumption inherent to the species tree model (i.e., the coalescent model assumes selective neutrality). Given these two concerns, we recognize that there is more overall nodal support for the concatenated estimate than for the species tree estimate (Fig. 1). Additionally, those nodes that are supported in the former are likely attributable to hidden support among genes (Gatesy and Baker, 2005), rather than evidence of anomalously branched gene genealogies, which we do not expect given the nature of our taxon sub-sampling and the depth of the phylogeny being estimated. Moreover, other causes of topological bias (introgression, gene duplication/extinction) also seem unlikely candidates to mislead us in this case, as multiple genes would likely need to share the same aberrant topologies in order for a sufficient number of substitutions to accrue across the concatenated dataset. Therefore, we include nodal support from the concatenated estimate as real support (or evidence rejecting) the taxonomic groupings in focus.

4.1. Tests of monophyly

Since neither concatenation nor species tree analyses are completely appropriate for our data, we quantified the support in the data for the taxonomic hypotheses on a gene by gene basis. We employed two techniques: filtering posterior topologies for trees containing groups in focus, and comparing the marginal likelihood estimates of positively and negatively constrained topologies using Bayes Factors. While we drew no strong conclusions from filtering the gene tree topology posteriors (Table 3), the Bayes Factor-based tests of monophyly provide results that are relatively clear in their interpretation. The relative power of the Bayes Factors is correlated with the number of segregating sites. On a locus-by-locus basis, we find little support for the taxonomic groupings of the earth snakes or the crayfish snakes. Rather, our data follow that of Alfaro and Arnold (2001) in suggesting that these groups are unnatural paraphyletic (in the case of *Virginia*) or polyphyletic (in *Regina*) assemblages. In addition, there was a strong conflict in the measurable support for the *Liodytes* and *Seminatrix* clade, with four of six genes supporting the relationship and one (MC1R) rejecting it. Incorporating the stepping stone sampling marginal likelihood estimates into Bayes Factor analyses provides a useful, straightforward technique to test phylogenetic hypotheses when sampled loci have less-than-ideal variability, leading to sub-optimally estimated gene trees.

4.2. Gene sampling

Results of Bayes Factor-based tests of monophyly were generally consistent across genes, except for the MC1R gene. For 3 of 6 tests, results from MC1R were opposite of that (i.e., rejected where others favored, and vice versa) of most other genes. This gene also exhibited an anomalous pattern when phase resolution was estimated; a pattern inconsistent with coalescent-based anomalous lineage sorting, given the depth of phylogeny being investigated. This result is also inconsistent with the findings of de Queiroz

et al. (2002), which placed *Adelophis* deep within *Thamnophis*, rather than related to either *Nerodia* or *Storeria*, which is exhibited by the MC1R phase resolution estimation in this study. Therefore, as a qualitative measure of its contribution to the multi-locus analyses, we re-estimated the concatenated phylogeny and species tree, excluding the MC1R data. Interestingly, topologies changed and overall support (average BPP across all nodes) improved in both analyses, and *Virginia* changed from a well-supported paraphyletic pair (where *V. striatula* is sister to *V. valeriae*, *Clonophis* and “*Liodytes*”) to a well-supported polyphyletic pair, where each is sister to a different clade of semi-fossorial snakes (Fig. 2). Though relatively easy to amplify and sequence, we would recommend that this gene be used with caution in phylogenetic and phylogeographic studies without incorporation of a more robust understanding of its evolution.

4.3. Evolutionary and taxonomic implications

Rossman (1963) described the crayfish snakes as sharing many morphological characteristics but displaying two distinct types: the pair with more standard dentition, *Regina grahamii* and *R. septemvittata*, which feed on recently molted crayfish, and the more extremely derived type (*R. alleni* and *R. rigida*) with chisel-like, kinetic teeth and specialized feeding behavior (Franz, 1977; Godley, 1980; Myer, 1987). Our data lend no support to the former type as a valid taxonomic group; however there was no outright rejection based on posterior probability. With morphological and allozyme-based evidence (Lawson, 1985) supporting this group, we are hesitant to suggest that they have independently evolved along ecological and morphological pathways without further study. Further, if the relationship in the concatenated estimate including MC1R is accurate (Fig. 1a), their similarities may represent shared ancestral characters. The latter group is supported, but with the inclusion of the black swamp snake (*Seminatrix pygaea*) as sister to *R. rigida* by both concatenated phylogenetic estimates. Interestingly, this relationship indicates a shift away from a specialized feeding ecology, and accompanying morphology, to a generalized diet in the swamp snake, which include amphibians, fish, and a variety of invertebrates (Gibbons and Dorcas, 2004). Interestingly, Manjarrez (2005) noted that a population of *Thamnophis melanogaster* specialized in preying upon soft-bodied crayfish, suggesting that there are as many as four independent origins of this behavior among thamnophiine snakes.

The earth snakes are represented by two small, gray, fossorial species, with largely overlapping ranges and subsisting on earthworms (Conant and Collins, 1991). Neither our nor the previous allozyme study support monophyly of this group, though, similar to the abovementioned case, the concatenated analysis including MC1R (Fig. 1a) suggests that they may share ancestral characters as basal members of the clade containing *Clonophis* and “*Liodytes*.” These findings highlight convergent evolution in feeding strategy similar to that observed in other natricine snakes (e.g., Hibbitts and Fitzgerald, 2005; Vincent et al., 2009).

Table 3

Lines of evidence supporting or rejecting the six monophyletic groupings. Proportions of posteriors are the ranges of the proportions of distribution of topologies among gene trees estimated.

Taxonomic grouping	Species tree	Concatenation	Gene tree support	Proportions of posteriors	BF constraint tests
“Earth snakes”	No support	Reject	2 Reject, 0 support	0–0.03	4 Reject, 0 support
“Crayfish snakes”	Reject	Reject	3 Reject, 0 support	0 in all genes	5 Reject
<i>Liodytes</i>	Reject	Reject	1 Reject, 0 support	0–0.11	2 Reject, 2 support
<i>Liodytes</i> + <i>Seminatrix</i>	No support	Support	0 Reject, 0 support	0.007–0.13	1 Reject, 4 support
<i>R. grahamii</i> + <i>R. septemvittata</i>	No support	No support	0 Reject, 0 support	0–0.08	4 Reject, 0 support
<i>Storeria</i>	Support	Support	0 Reject, 1 support	0.08–0.99	0 Reject, 4 support

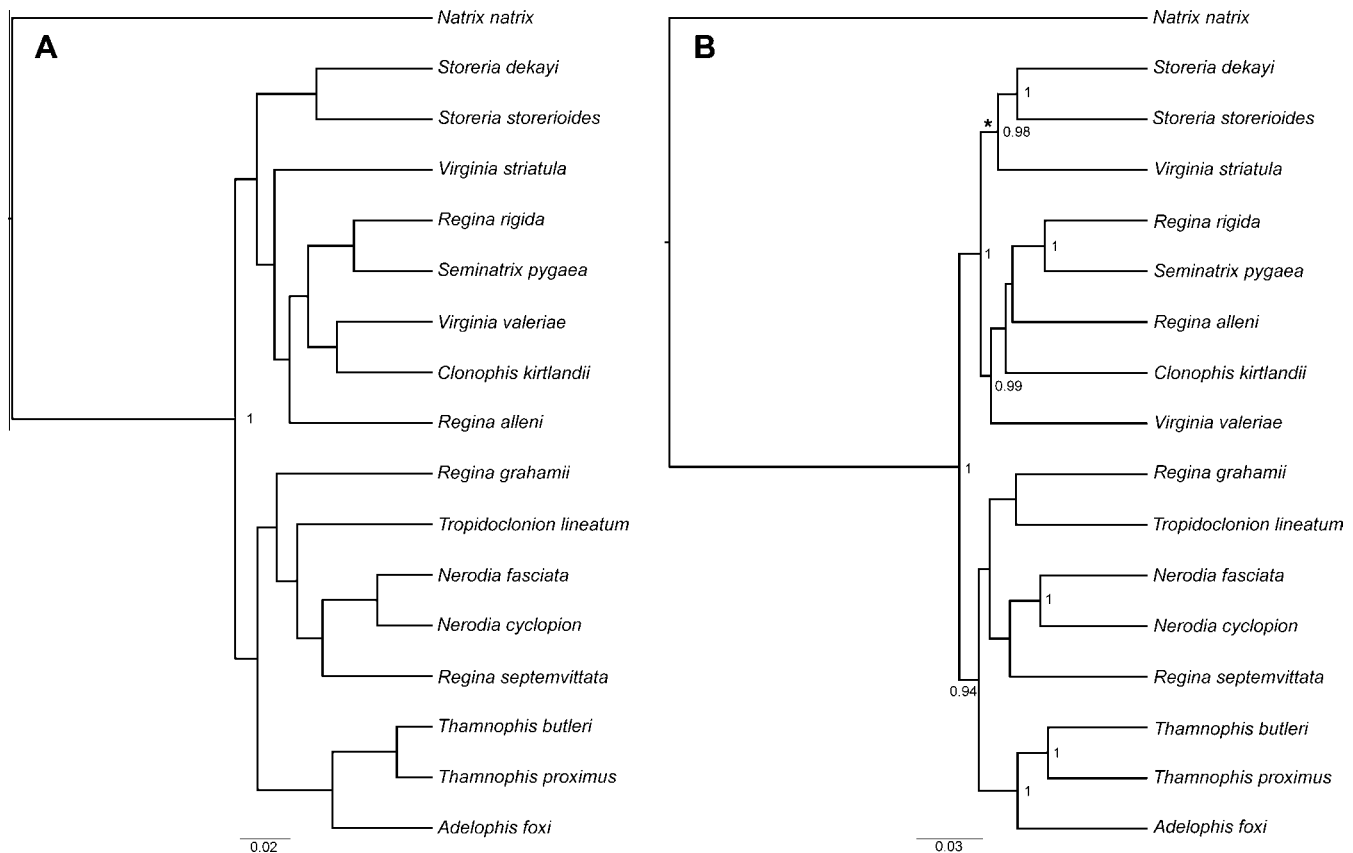


Fig. 2. Multi-locus Bayesian maximum clade credibility estimates with MC1R data excluded. (A) Multi-species coalescent; (B) concatenated phylogeny. *Indicates node in conflict (BPP > 0.95) with analysis including MC1R.

Our data lend support to the previous argument that crayfish predation arose more than once among Thamnophiini (Table 2), and the phylogenetic estimate, where overlapping, was in agreement with the estimates presented by Alfaro and Arnold (2001), and de Queiroz et al. (2002). Interestingly, *Adelophis* is only supported by the mitochondrial and concatenated estimates as being nested within *Thamnophis*. However, there no supported topologies among gene trees that disagree with this relationship, and the position of *Adelophis foxi* is deep within *Thamnophis* shown by de Queiroz et al. (2002). Here, we conclude that this observation is a result of poorly estimated gene trees rather than coalescent stochasticity or introgression, though incorporation of the rare *Adelophis copei* may improve our placement of this genus among the thamnophiine snakes. Some relationships among taxa within this group remain unresolved; advances in genomic data collection and analytical methodology will facilitate investigation into relationships among Thamnophiini, allowing for more robust models of divergence and diversification within this group. We support the resurrection of the genus *Liodytes* for the currently recognized *Regina alleni* and *rigida*, with the reclassification of *Seminatrix pygaea* to *Liodytes* as well. Both generic names are available for the clade; *Liodytes* (Cope, 1885) takes precedence over *Seminatrix* (Cope, 1895). In the case of the earth snakes, there was virtually no support for but ample rejection of their monophyly. Based this evidence, we suggest the resurrection of the genus *Haldea* (Baird and Girard, 1853) for the currently recognized *Virginia striatula*.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ympev.2013.04.028>.

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