

Molecular Systematics and Evolution of *Regina* and the Thamnophiine Snakes

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Snakes of the tribe Thamnophiini represent an ecologically important component of the herpetofauna in a range of habitats across North America. Thamnophiines are the best-studied colubrids, yet little is known of their systematic relationships. A molecular phylogenetic study of 32 thamnophiine species using three complete mitochondrial genes (cytochrome *b*, NADH dehydrogenase subunit 2, and 12S ribosomal DNA) recovered a well-supported phylogeny with three major clades: a garter snake group, a water snake group, and a novel semifossorial group. The historically contentious genus *Regina*, which contains the crayfish-eating snakes, is polyphyletic. The phylogeographic pattern of *Thamnophis* is consistent with an hypothesis of at least one invasion of northern North America from Mexico.

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INTRODUCTION

The New World natricines (Tribe Thamnophiini) are a diverse group of colubrid snakes comprising nine genera and roughly 50 species. The best known of these are the garter snakes (*Thamnophis*), the water snakes (*Nerodia*), and the crayfish snakes (*Regina*), with most of the other genera containing small-bodied, secretive species. Members are ecologically varied in diet and lifestyle. Species take a variety of vertebrate and invertebrate prey, including fish, amphibians, earthworms, mammals, crabs, and crayfish, and inhabit aquatic, terrestrial, and semifossorial environments (Arnold, 1981). Numerous studies, ranging from ecology and population biology (Siegel and Ford, 1987; Rossman *et al.*, 1996) to functional morphology (Cundall and Gans, 1979; Cundall, 1983; Kelley *et al.*, 1997), have been conducted on tribe members, particularly on the most diverse and conspicuous genera, *Thamnophis* and *Nerodia*.

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Despite the history of inquiry into the biology of these organisms, very little is known about the phylogenetic history of the group. Whereas they have been recognized as a monophyletic assemblage (George and Dessauer, 1970; Mao and Dessauer, 1971; Rossman and Eberle, 1977; Schwaner and Dessauer, 1982) relationships within and among genera are largely unresolved. Morphological and molecular evidence suggests a close relationship between *Thamnophis* and *Nerodia*; however, the relationship of these genera to other taxa is unclear (Varkey, 1979; Lawson, 1987). The relationships among the crayfish snakes, genus *Regina*, have been long disputed (Price, 1982, 1983; Rossman, 1985; Lawson, 1987). Relationships among the other genera remain almost completely unknown. A phylogeny for these snakes would illuminate the biogeography of these important North American colubrids and provide a framework for future comparative study of ecology and evolution within the tribe.

The goals of this study were to elucidate the phylogenetic relationships of North American natricine snakes and examine the evolution of various mitochondrial genes to assess their phylogenetic utility. A molecular phylogeny for 32 natricine taxa is proposed based on complete nucleotide sequences from three mitochondrial genes.

MATERIALS AND METHODS

Sampling and PCR

Twenty-eight species of the snake tribe Thamnophiini were sampled, including representatives of all genera except *Adelophis* (a rare Mexican genus containing 2 species) (Table 1). One European (*Natrix maura*) and two Asian (*Amphiesma sauteri*, *Rhabdophis nuchalis*) natricine snakes were used as outgroups. Old World Natricines are considered the closest relatives of the thamnophiines (Mao and Dessauer, 1971; Rossman and Eberle, 1977; Schwaner and Dessauer, 1982). Total genomic DNA was extracted from most taxa with the PureGene extraction kit and protocol (Gentra Systems). *Amphiesma* and *Rhabdophis* DNA was ex-

TABLE 1
Materials Examined in This Study

Genus	Species	Locality	Voucher Number
<i>Nerodia</i>	<i>cyclopion</i>	Baton Rouge Parish, LA	SJA 7995B
<i>Nerodia</i>	<i>erythrogaster</i>	Lonoke Co., AR	SJA 6512
<i>Nerodia</i>	<i>fasciata</i>	TX	MEA 501
<i>Nerodia</i>	<i>floridana</i>	Alachua Co., FL	MEA 502
<i>Nerodia</i>	<i>harteri</i>	Palo Pinto Co., TX	SJA 1166
<i>Nerodia</i>	<i>rhombifer</i>	Lonoke Co., AR	SJA 6592
<i>Nerodia</i>	<i>sipedon</i>	Cook Co., IL	MEA 503
<i>Natrix</i>	<i>taxispilota</i>	Hillsborough Co., FL	SJA 6689
<i>Regina</i>	<i>alleni</i>	Alachua Co., FL	MEA 504
<i>Regina</i>	<i>grahami</i>	TX	MEA 505
<i>Regina</i>	<i>rigida</i>	Franklin Co., FL	UF113334
<i>Regina</i>	<i>septemvittata</i>	Cook Co., IL	FMNH 257458
<i>Seminatrix</i>	<i>pygaea</i>	Alachua Co., FL	SJA 7787
<i>Storeria</i>	<i>dekayi</i>	Cook Co., IL	SJA3931
<i>Storeria</i>	<i>occipitamaculata</i>	Macon Co., NC	SJA 6064
<i>Thamnophis</i>	<i>atratus</i>	Mendocino Co., CA	SJA 869
<i>Thamnophis</i>	<i>butleri</i>	Milwaukee Co., WI	HKV 32234
<i>Thamnophis</i>	<i>cyrtopsis</i>	Presidio County, TX	FMNH257124
<i>Thamnophis</i>	<i>elegans</i>	Lassen Co., CA	SJA 868
<i>Thamnophis</i>	<i>marcianus</i>	Guadalupe Co., NM	MEA 506
<i>Thamnophis</i>	<i>ordinooides</i>	Del Norte Co., CA	SJA 7826
<i>Thamnophis</i>	<i>proximus</i>	Commercial wholesaler	MEA 507
<i>Thamnophis</i>	<i>radix</i>	Cook Co., IL	MEA 508
<i>Thamnophis</i>	<i>sirtalis infernalis</i>	Humboldt Co., CA	SJA 4545
<i>Thamnophis</i>	<i>s. parietalis</i>	Commercial wholesaler	MEA 509
<i>Tropidoclonion</i>	<i>lineatum</i>	Russell Co., KS	SJA 3932
<i>Virginia</i>	<i>striatula</i>	Wake Co., NC	SJA 7735
<i>Virginia</i>	<i>striatula</i>	Harrison Co., MS	SJA 8002B
<i>Amphiesma</i>	<i>sauteri</i>	Hongya Xian., Sichuan, China	FMNH 232808
<i>Rhabdophis</i>	<i>nuchalis</i>	Hongya Xian., Sichuan, China	HKV 36838
<i>Natrix</i>	<i>maura</i>	St. Laurent le Minier, Dept. du Gard, France	MEA 510

Note. FMNH, Field Museum of Natural History; UF, University of Florida; HKV, Harold K. Voris (tissue No.); MEA, Michael Edward Alfaro (tissue No.); SJA, Stevan J. Arnold (tissue No.).

tracted with phenol/chloroform after an overnight digest in proteinase K extraction buffer (100 mM tris, 10 mM Na₂-EDTA, 100 mM NaCl, 1% SDS, 10 mg/ml dithiothreitol, and 0.03 mg proteinase K). Extractions

were quantified by UV spectroscopy and diluted to 50 ng/ μ l. Each gene was amplified with two primer pairs (Table 2). Standard PCR protocols were followed. PCR reactions were performed in 25- μ l volumes with an

TABLE 2
Primers Used in This Study

Gene	Primer	Sequence (5'–3')	Position
Cytb	LGlu	TGATCTGAAAAACCACCGTTGTA	14889–14911
Cytb	H15544	AATGGGATTTTGTCAATGTCTGA	15541–15564
Cytb	L15446	CCAACCCTAACACGATTCTTTGC	15421–15443
Cytb	Hpro	TTAAGTTAAAATACTGGCTTTGG	16139–16161
ND2	L49	CTATTATGCGCCACCCATCAAT	5291–5313
ND2	H50	CGGTGCTATTTTTAGTGTTGCTA	5400–5422
12S	L12S3	AAAGCATAGCACTGAAAATGC	21–41
12S	H12S6	GGTTATTAGACAGGCTCCTCTA	607–628
12S	L12S4	GGTGTGAAGTACCGTCAAGTC	582–602
12S	H12S8	CGAGTGTAGGTCGAGTGCTTTG	894–916

Note. For Cytb and 12S, two primer sets anchored in flanking tRNA regions were used to obtain complete sequence from both light and heavy strands. For ND2, new internal primers were made to complement ND2-1 and ND2-2. Position is in reference to *Dinodon semicarinatus* (GenBank Accession No. NC 001945).

annealing temperature of 46–52°C with an MJ Research thermocycler. PCR products were purified with the GeneClean protocol (Bio 101). Sequencing reactions were performed with PRISM Dye Terminator Cycle Sequencing Ready Reaction Kits or the PRISM dRhodamine Kits, following the manufacturer's protocols (P. E. Biosystems). Reactions were purified with ethanol precipitation according to the manufacturer's protocol and then electrophoresed with an ABI 377 automated sequencer (P. E. Biosystems).

Mitochondrial DNA Sequence Data

Sequence from all three genes was trimmed to the size of the smallest fragment that was successfully sequenced to minimize the amount of missing data that was introduced to the data matrix. The cytochrome *b* (Cytb) primers amplified a 1227-bp region that spanned all of Cytb and the adjacent tRNAs. This sequence was trimmed to 1083 bp for analysis (GenBank Accession No. AF402905-402936). The NADH dehydrogenase subunit 2 (ND2) primers amplified a 1056-bp region, including portions of adjacent tRNAs, 1021 bp of which were used in this study (GenBank Accession No. AF384824-384855). The 12S ribosomal DNA (12S) primers amplified a region approximately 976 bp long that was trimmed to 935 bp in our analysis (GenBank Accession No. AF402622-402653). We used a mitochondrial genome sequence from a colubrid snake, *Dinodon semicarinatus* (GenBank Accession No. NC001945), to construct primers and to aid initial alignments. Protein-coding sequences were aligned by eye with Sequencher 3.1 (GeneCodes, 1998) and PAUP* (Swofford, 1998).

For 12S rRNA, secondary structure models for mammals (Springer and Douzery, 1996; Gutell, 1994) and birds (Mindell *et al.*, 1997) were used as a template for the construction of a secondary structure model for snakes. Domain III of our model was further refined by comparison to proposed secondary structures for a wide range of invertebrates and vertebrates (Hickson *et al.*, 1996). We created an initial snake alignment by identifying regions of the molecule that were conserved among mammals, birds, and snakes. Two regions of the molecule were unalignable to previous models on the basis of sequence similarity and were examined in more detail. These regions correspond to bases peripheral to stems 6 and 18 in the Springer and Douzery (1996) model. As a first step to determining the structure of these ambiguously alignable regions, we used the program MFOLD 3.0 (Zuker *et al.*, 1998) to predict the secondary structure for each of these regions according to a thermodynamic model that minimizes free energy. To anchor secondary structure calculations, we inputted sequence from the conserved stem nearest the ambiguous region along with sequence from the ambiguous region itself into MFOLD and forced this stem to pair. This procedure was repeated for all study taxa.

In both regions multiple secondary structure models were possible for certain taxa. We chose the folding that was recovered for the greatest number of taxa to use as the working hypothesis of the secondary structure for that region. Ambiguously aligned regions, usually corresponding to loop positions, were excluded from all analyses.

Preliminary Data Exploration

Heterogeneity in base composition has been shown to affect phylogenetic reconstruction (Galtier and Gouy, 1998; Lockhart *et al.*, 1994; Yang and Roberts, 1995). To determine whether base heterogeneity was present in our dataset, Cytb and ND2 sequences were tested at each codon position and 12S sequences were tested at stems and loops by means of a χ^2 analysis of base frequencies across taxa. High levels of base substitution, "saturation," have been shown to impair phylogenetic reconstruction (Blouin *et al.*, 1998; Halaných *et al.*, 1999). To visually assess saturation of gene partitions, the numbers of transitions and transversions at first, second, and third codon positions for ND2 and Cytb and at stem and loop regions for 12S were plotted against Jukes–Cantor genetic distance (Jukes and Cantor, 1969).

Congruence Tests

Although all three genes examined in this study come from the mitochondrion and thus would be expected to reflect a shared evolutionary history, it is possible that evolutionary processes among genes have differed enough that there would be apparent conflict in the phylogenetic signal among genes (Bull *et al.*, 1993). The incongruence length difference (ILD) test (Farris *et al.*, 1995), as implemented in PAUP* (Swofford, 1998), was used to determine whether significant conflict existed among the three data partitions (Cytb, ND2, and 12S). The heuristic search option, with 10 random addition sequence replicates and 300 total replicates, was used to generate the null distribution for these tests. Following Cunningham (1997) invariant sites were excluded and a significance level of 0.01 was adopted for this test. None of the partitions were found to significantly conflict with one another. Pairwise tests between the protein-coding genes and 12S rRNA were most nearly significant (Cytb vs 12S: $P = 0.06$; ND2 vs 12S: $P = 0.04$). Since the ILD test indicated that there was not significant conflict among any of the partitions, we combined our data for phylogenetic analyses.

Parsimony Analyses

Maximum-parsimony (MP) analyses were conducted with PAUP* (Swofford, 1998) with the heuristic search option with 100 random addition sequence replicates and tree bisection–reconnection (TBR) branch swapping. Plots of substitution number versus genetic dis-

TABLE 3
Base Frequencies for Three Mitochondrial Genes in Natricine Snakes

Species	Cytb											
	1st position				2nd position				3rd position			
	A	C	G	T	A	C	G	T	A	C	G	T
<i>Amphiesma saurteri</i>	0.33	0.22	0.18	0.27	0.19	0.30	0.11	0.40	0.43	0.31	0.03	0.23
<i>Natrix maura</i>	0.32	0.25	0.19	0.24	0.19	0.29	0.12	0.40	0.40	0.42	0.04	0.15
<i>Rhabdophis nuchalis</i>	0.33	0.23	0.17	0.27	0.20	0.29	0.11	0.40	0.41	0.36	0.04	0.19
Thamnophiini (mean)	0.32	0.24	0.18	0.25	0.19	0.29	0.12	0.40	0.43	0.37	0.03	0.17
Species	ND2											
	1st position				2nd position				3rd position			
	A	C	G	T	A	C	G	T	A	C	G	T
<i>Amphiesma saurteri</i>	0.44	0.24	0.13	0.18	0.17	0.36	0.09	0.38	0.54	0.28	0.04	0.14
<i>Natrix maura</i>	0.43	0.25	0.13	0.18	0.15	0.40	0.09	0.36	0.54	0.30	0.04	0.12
<i>Rhabdophis nuchalis</i>	0.47	0.21	0.16	0.16	0.18	0.38	0.10	0.35	0.51	0.28	0.05	0.15
Thamnophiini (mean)	0.47	0.24	0.13	0.16	0.16	0.38	0.09	0.37	0.54	0.33	0.03	0.10
Species	12S											
	Stems				Loops							
	A	C	G	T	A	C	G	T	A	C	G	T
<i>Amphiesma saurteri</i>	0.24	0.26	0.26	0.23	0.47	0.24	0.13	0.16				
<i>Natrix maura</i>	0.24	0.26	0.27	0.23	0.46	0.25	0.13	0.16				
<i>Rhabdophis nuchalis</i>	0.24	0.27	0.26	0.23	0.49	0.20	0.13	0.18				
Thamnophiini (mean)	0.24	0.26	0.26	0.24	0.47	0.24	0.13	0.16				

Note. Data for three Old World natricines and means for the New World tribe are shown. Within the thamnophiines, base frequencies were generally similar to each other, showing the most variation within the garter snakes at Cytb and ND2 third positions.

tance (not shown) suggested that some partitions were saturated. We explored three weighting schemes to account for saturation: equal weights for all partitions, weighting of third position transitions one half that of other base changes at other positions, and exclusion of third position transitions. To estimate nodal support, bootstrapping (Felsenstein, 1985) was employed with 1000 replicates and 100 random addition sequence replicates with TBR branch swapping. Gaps in 12S data were treated as missing data.

Likelihood Analysis

Maximum-likelihood (ML) searches were performed with PAUP* (Swofford, 1998). In general, the more parameter-rich a likelihood model is, the better it will fit the data. However, this increased fit comes at the expense of explanatory power, so it is important to consider the degree to which additional parameters improve the likelihood (Huelsenbeck and Crandall, 1997; Posada and Crandall, 1998). To justify an appropriate model for likelihood analysis we used the program MODELTEST 3.0 (Posada and Crandall, 1998), which automatically performs a series of likelihood ratio tests (Huelsenbeck and Crandall, 1997; Huelsenbeck and Rannala, 1997) on nested likelihood models.

MODELTEST results indicated that a general time

reversible (GTR) model with variable rates and invariant sites was justified by our data. We performed a heuristic search under this model using PAUP* (Swofford, 1998). Twenty-five random addition sequences with TBR branch swapping were performed during this search. To estimate nodal support, bootstrapping was performed under the likelihood model with 100 replicates. To decrease time for likelihood bootstrapping, starting trees were generated by neighbor-joining with the maximum-likelihood distance matrix. For all likelihood analyses, two taxa missing approximately 500 bp of sequence (*R. nuchalis* and *Nerodia harteri*) were excluded from analysis to save computational time.

RESULTS

Both parsimony and likelihood analyses produced well-resolved phylogenies that were largely congruent with one another. In both analyses, monophyly of *Thamnophis* was supported, whereas *Nerodia* was found to be paraphyletic with respect to other thamnophiines and *Regina* was polyphyletic. Below, these results are discussed in further detail.

TABLE 4
Variable and Informative Sites for Gene Partitions in Thamnophiine Snakes

	CytB				ND2				12S		
	1st	2nd	3rd	Total	1st	2nd	3rd	Total	Stems	Loops	Total
% var.	29	12	93	44	39	19	82	46	19	28	23
% inf.	70	70	80	77	56	51	78	69	48	68	59

Note. % var, the percentage of variable sites at a position; % inf., the percentage of variable sites that are informative under the parsimony criterion.

Sequence Divergence and Base Frequency

We found substantial differences in base composition among genes and in codon and stems/loop partitions within genes (Table 3). Within individual partitions, taxa were largely similar in base composition at all positions except at Cytb third positions. χ^2 tests failed to reject the hypothesis of base homogeneity among taxa for all genes and partitions.

Divergences in Cytb ranged from p (uncorrected genetic difference) = 0.013–0.129 for the ingroup to a maximum of p = 0.198 from ingroup to outgroup. Divergences in ND2 were slightly higher, ranging from p = 0.029–0.136 within the ingroup and up to p = 0.209 to the outgroup. 12S divergences were much lower than the protein-coding genes in this study, ranging from p = 0.009–0.050 for the ingroup and p = 0.092 from ingroup to outgroup.

Variability of sites within partitions is shown in Table 4. For both protein-coding genes, codon position 2 showed the lowest variability and position 3 the highest, as expected. Sites at all three positions in ND2 were more variable than those in Cytb, although they were also more unique: ND2 had a lower proportion of parsimony-informative sites (Table 4). In 12S, stem regions contained a relatively small proportion of variable sites and fewer potentially informative sites compared to loop regions.

Saturation

In both protein-coding genes, all changes at first and second positions and transversions at third positions increased in a near linear fashion with increasing genetic distance (not shown). Third position transitions in both genes appeared to level off at higher divergences, particularly among outgroup to ingroup comparisons. We interpreted this as evidence for saturation in these partitions. There was no evidence of saturation within 12S.

12S Secondary Structure Model

Figure 1 shows the proposed model of 12S RNA secondary structure for thamnophiine snakes, following the nomenclature of Springer and Douzery (1996). On the basis of our data, we identified regions in do-

mains I and II that appear to have undergone substantial structural change relative to other vertebrates.

Region 1. This region was approximately 12–14 bases shorter than homologous regions in mammals and birds and was difficult to align to previously published models. The most frequently recovered structure by MFOLD for this region was dominated by a single large multiloop distal to stem 6. Peripheral to this multiloop were three stem regions, two of which on the basis of position and sequence similarity we homologized with stems 9 and 10 of Springer and Douzery (1996). A single stem is present in a position analogous to stems 7 and 8 in other vertebrates, suggesting that one of these helices or the loop between them has been lost in snakes. In addition, the terminal loop peripheral to this novel stem (stem 7 in Fig. 1) is approximately three times larger than the loop peripheral to stem 8 in mammals and birds.

Region 2. Bases peripheral to stem 18 were difficult to align with mammal and bird 12S models. MFOLD predicted that there were only two stems within this region (tentatively homologized with regions 19 and 21 of the Springer–Douzery model on the basis of position and sequence similarity) instead of the three found in other vertebrates.

The 12S model presented here is a preliminary hypothesis for the structure of this molecule in thamnophiine snakes. Due to the relatively low taxonomic sampling level of the phylogenetic analyses in this study, there are few examples of positional covariation indicative of compensatory change within stems in our data set. Compensatory change is regarded the best evidence for verification of secondary structure (Gutell *et al.*, 1994; Springer *et al.*, 1995; Springer and Douzery, 1996) and future work will focus on broad-scale sampling of snakes to generate the data necessary to refine this model.

Parsimony Analysis

Under a search employing equal weights of characters for all positions, two most parsimonious trees (MPTs) were recovered (Fig. 2). The topologies differed only in the position of *Nerodia erythrogaster*, placing it

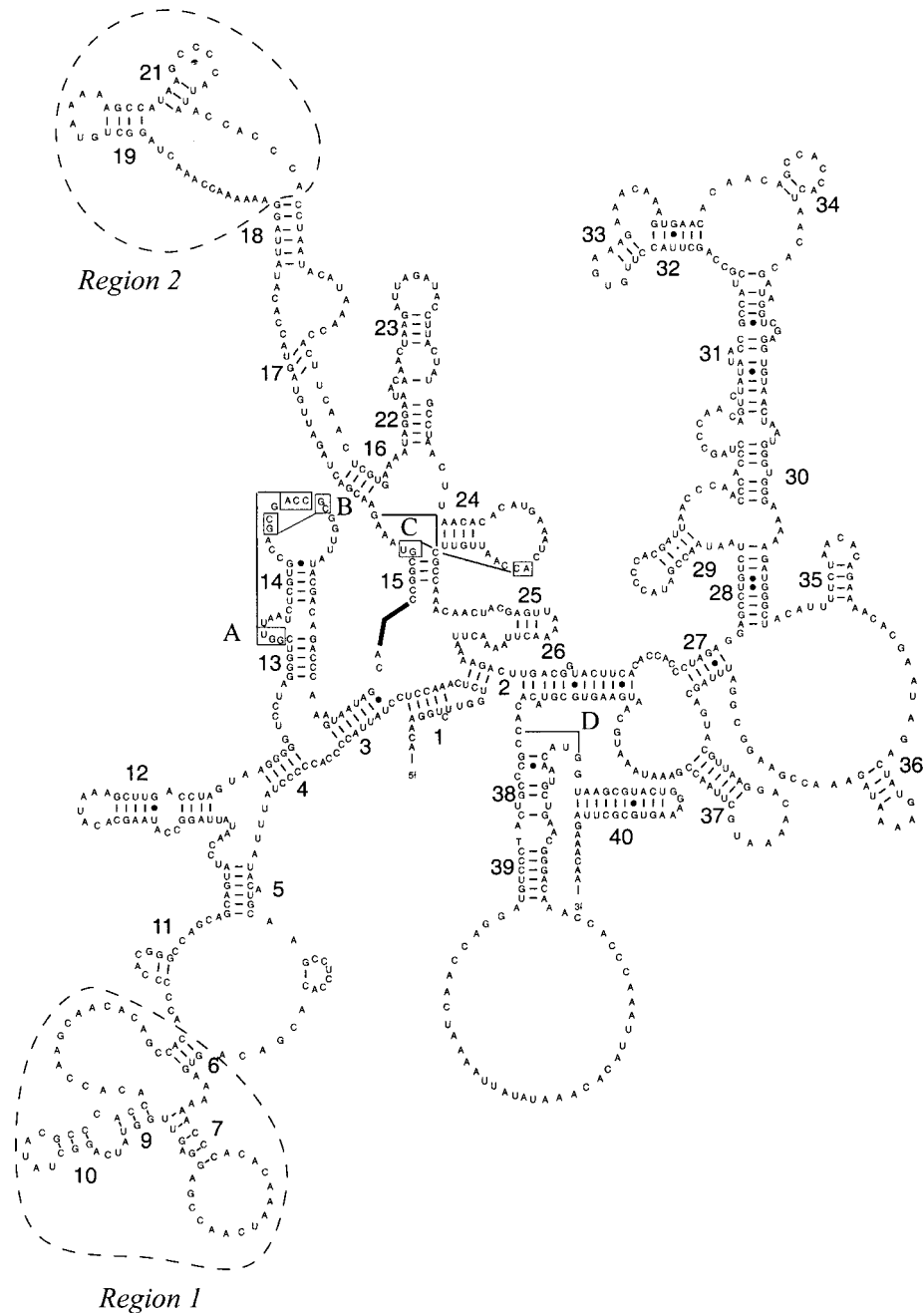


FIG. 1. Snake 12S ribosomal RNA model. Shown is the secondary structure model for thamnophiine 12S RNA illustrated with *Thamnophis marcianus*. Stem numbering follows Springer and Douzery (1996). Dashes indicate base pairing; dots indicate U-G (non-canonical) base pairs. Uppercase letters refer to predicted tertiary interactions (from Gutell, 1994). Dotted lines indicate two regions that could not be aligned unambiguously to either mammal (Springer and Douzery, 1996) or bird (Mindell *et al.*, 1997) models.

as either the sister taxon to *N. taxispilota* + *N. rhombifer* or *N. fasciata* + (*N. sipedon* + *N. harteri*). Three major clades were recovered, herein referred to as the semifossorial group, the water snake group, and the garter snake group (Fig. 2). The semifossorial clade formed the basal thamnophiine group and was composed of seven taxa: *Virginia*, *Storeria* and *Clonophis*, truly semifossorial species; *Seminatrix*, a semiaquatic

species; and two of the crayfish snakes, *Regina alleni* and *R. rigida*. Weighting generally had little effect on topology. Downweighting of third position transitions produced a single most parsimonious tree that was identical to the equal-weights MPT with *N. erythrogaster* as the sister group to *N. fasciata* + (*N. sipedon* + *N. harteri*). Elimination of third position transitions produced six MPTs. The consensus of these trees was

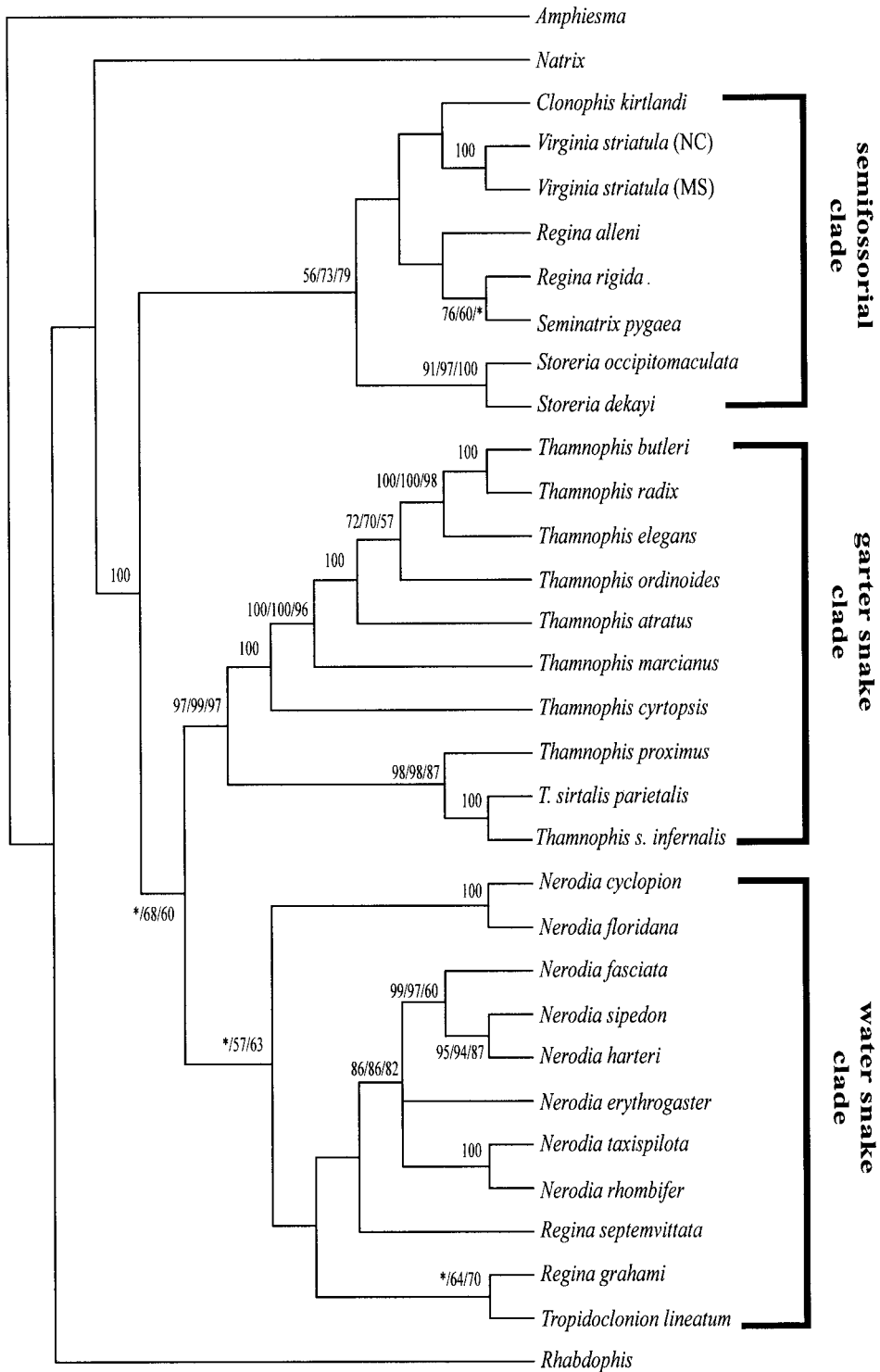


FIG. 2. Consensus of two equally most parsimonious trees. Tree length = 3665 steps; Consistency Index = 0.428; Retention Index = 0.458. Numbers at nodes represent percentage of bootstrap replicates supporting that node under three different weighting schemes: equal weights, third position transitions downweighted by one half relative to other changes, and third position transitions excluded. Three main clades are recovered, although bootstrap support is generally weak for the water snake clade. The genus *Regina* is polyphyletic and *Nerodia* is paraphyletic with respect to other thamnophiines.

congruent with the equal-weights consensus MPT. However, there was a substantial loss of resolution in the semifossorial clade with *Clonophis*, *R. alleni*, *R. rigida*, *Seminatrix*, and *Virginia* forming an unresolved sister group to *Storeria*. Similarly, relationships at the base of the water snake clade collapsed to a four-way polychotomy comprising *Nerodia cyclopion* + *N. floridana*, *Regina septemvittata*, *R. grahmi* + *Tropidoclonion lineatum*, and the rest of the members of *Nerodia*. Relationships within *Thamnophis* were not affected by choice of weighting scheme.

Within the novel semifossorial clade, bootstrap support for *Storeria* monophyly was strong under all weighting schemes. Our unweighted analysis also produced moderate support for a sister group relationship between *R. rigida* and *Seminatrix*; however, this support eroded under successively higher weighting schemes. Other relationships within the clade were not well supported by bootstrapping. Support for the semifossorial clade itself was poor under equal weights, but improved as weighting increased.

Bootstrap support across the garter snake group was high and largely unaffected by weighting scheme. The single exception concerned the clade containing *Thamnophis ordinoides* + more derived garter snakes, which received weak bootstrap support under the highest weighting scheme. The genus *Nerodia* was paraphyletic with respect to three taxa: *R. septemvittata*, *R. grahmi*, and *T. lineatum*. Bootstrap support was weak for the basal nodes of the water snake group and for the clade itself, although support for some of these nodes increased with weighting.

Likelihood Analysis

MODELTEST results indicated that a GTR model with rate matrix $R(a) = 0.7700$, $R(b) = 12.1829$, $R(c) = 1.4542$, $R(d) = 0.3531$, $R(e) = 12.8707$, a proportion of invariant sites equal to 0.8509, and an alpha parameter of 0.5168 for a gamma distribution of rates fit our data significantly better than all simpler models. A molecular clock was rejected for this model ($P < 0.001$).

A heuristic search under this model recovered a single ML tree (Fig. 3) found in 12 of 25 replicate random addition sequences. The ML topology was largely similar in topology to the MP trees. Three major clades were recovered with the same membership as found in the MP trees. As in the parsimony analysis, a clade composed of *R. alleni* + (*R. rigida*, *Seminatrix*) was recovered, although in the ML tree this formed the basal semifossorial clade. Bootstrap support was strong for the semifossorial clade and for *Storeria* monophyly and moderate for *R. alleni* + (*R. rigida*, *Seminatrix*). Topology of the garter snake group differed from that of the parsimony trees in placing *T. ordinoides* and *T. atratus* as sister taxa to each other rather than as successive sister taxa to *T. elegans* + (*T.*

butleri, *T. radix*). As in the parsimony analysis, bootstrap support was strong throughout this clade.

Relationships within the water snake group were also similar to those found in the MP trees. The same two lineages of *Nerodia* were recovered, and relationships between and within them were very similar, although the ML tree favors the placement of *N. erythrogaster* as sister to *N. fasciata* + *N. sipedon*. Once again, *Nerodia* paraphyly with respect to two *Regina* species and *Tropidoclonion* was observed, although in the ML tree the relative positions of *R. septemvittata* and *R. grahmi* + *Tropidoclonion* are swapped. Bootstrap support for the water snake clade was higher than for this clade under parsimony.

DISCUSSION

The current diversity of North American natricines is due to differentiation within three main thamnophiine lineages. Whereas monophyly of the garter snakes was strongly supported, our results suggest that other currently recognized genera, including *Regina* and *Nerodia*, are paraphyletic or polyphyletic with respect to other thamnophiines.

Gene Partitions

Cytb and ND2 appeared to evolve at similar rates within thamnophiines and showed signs of saturation only at third position transitions. However parsimony analysis revealed a striking difference in the phylogenetic performance of these partitions. Cytb recovered a larger proportion of the nodes in the combined MP tree than ND2 and bootstrap support for those nodes was also higher (results not shown). This difference in phylogenetic utility may be related to the distribution and number of parsimony-informative sites in the two genes (Table 4). Although more sites at first and second positions, and slightly more total sites, vary in ND2, the consequence of this apparent relaxation of functional constraint relative to Cytb is a higher proportion of uninformative singleton mutations.

By itself, 12S appeared to have less phylogenetic utility than either of the protein-coding genes examined in this study. 12S contributed the least number of parsimony-informative characters, both in absolute number of characters and in percentage of variable characters that were informative. Although this gene was initially chosen to complement the faster-evolving protein-coding genes by providing resolution at deeper nodes in the phylogeny, bootstrapping of the 12S data alone revealed strong support only for shallow nodes (not shown). Furthermore, the exclusion of 12S from the dataset had no effect on the topology of the tree recovered under parsimony, although bootstrap support for deeper nodes across the tree was lower with 12S excluded. For phylogenetic questions at this level in snakes, 12S alone does not appear to perform well

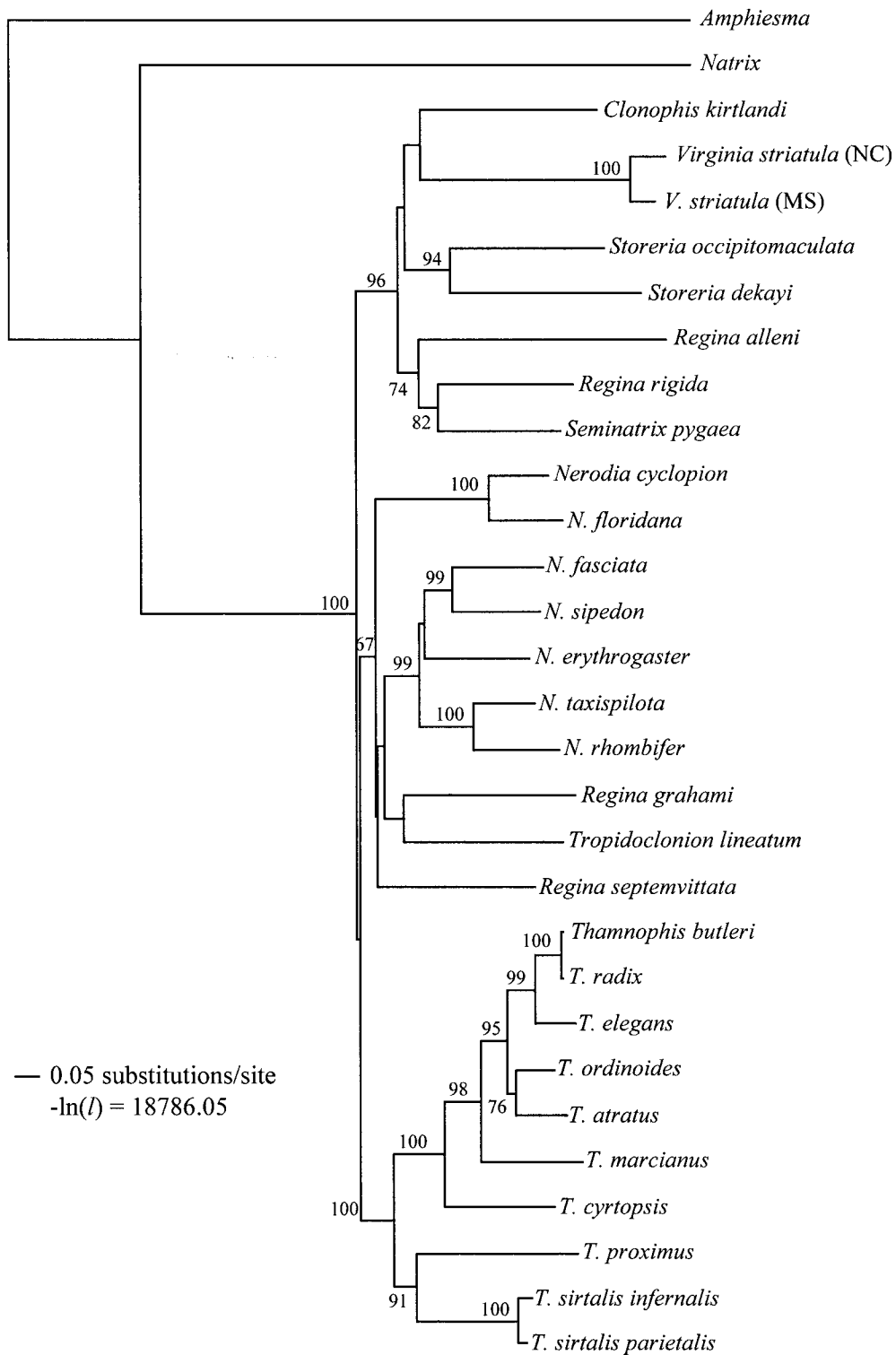


FIG. 3. Maximum-likelihood tree. Shown is the single most likely tree ($-\ln(l) = 18786.05$) recovered under a GTR model with invariant sites and rate heterogeneity among sites. Numbers at nodes represent percentage of bootstrap replicates supporting that node. Topologies of trees recovered under maximum-parsimony and maximum-likelihood are largely similar, although relationships within the semifossorial clade differ between the two types of analyses.

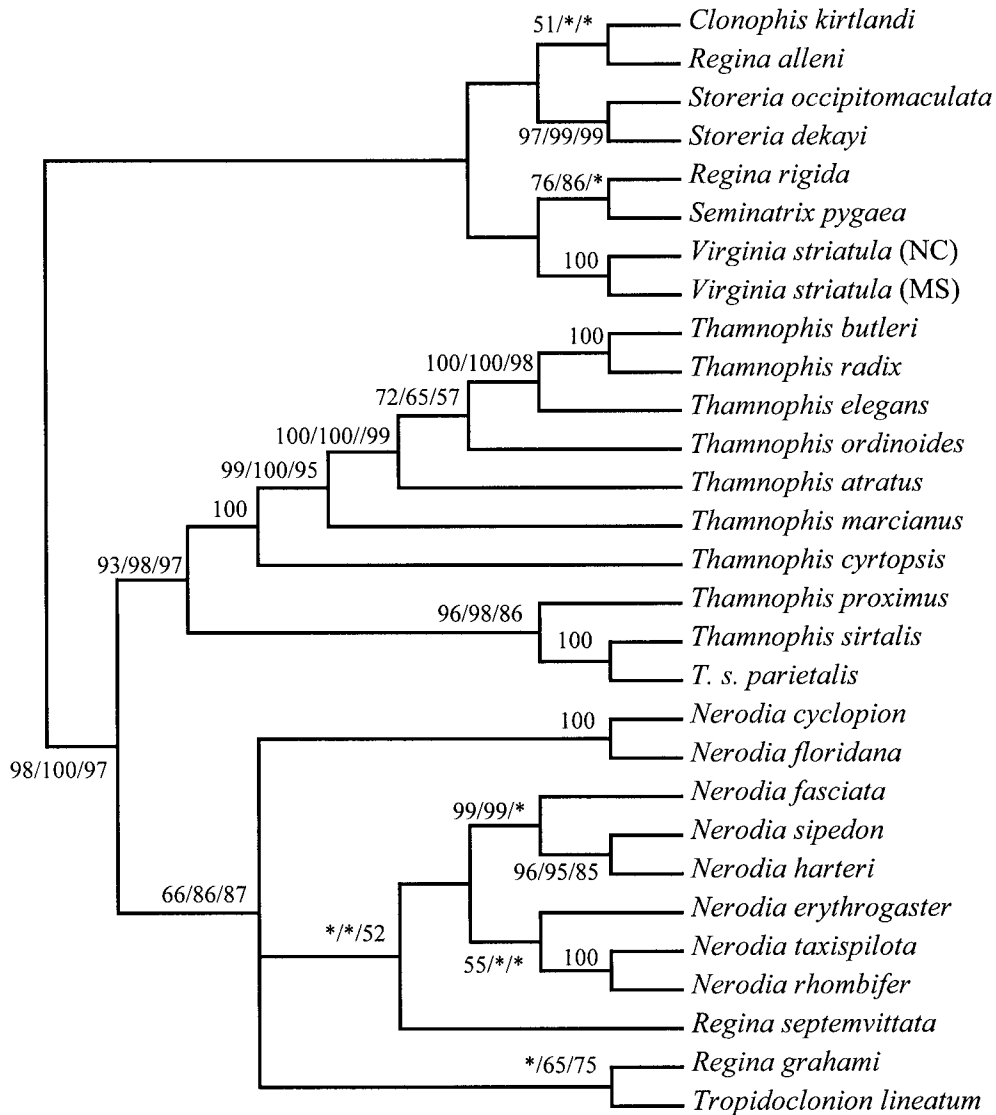


FIG. 4. Strict consensus of two equally most parsimonious unrooted networks of ingroup taxa. Tree length = 2795; Consistency Index = 0.438. Numbers at nodes represent percentage of bootstrap replicates supporting that node under three different weighting schemes: equal weights, third position transitions and loop transversions downweighted by one half, and third position transitions and loop transversions downweighted to 0. Asterisks indicate that bootstrap support for that node was less than 70 under that weighting scheme. Without outgroups, bootstrap support dramatically increases for the bipartition between the semifossorial group and the rest of the thamnophiines and increases for the bipartition between the water snakes clade and the rest of the thamnophiines. This result suggests that long branch attraction may be contributing to root instability in the rooted analysis.

compared to Cytb and ND2. However, it does appear to complement the faster-evolving genes by adding to bootstrap support at deeper levels in the tree.

Differences in Topology

The major differences between the MP and the ML trees were their hypotheses of relationships within the semifossorial clade and their estimates of support for the deeper nodes of the phylogeny. We suspected that long branch attraction (LBA) (Felsenstein, 1978; Huelsenbeck, 1997, 1998; Whiting *et al.*, 1997) could be

contributing to these differences in topology for two reasons. First, internodes connecting lineages are short relative to terminal branch length in at least two or three regions of the tree: within the semifossorial clade and at the base of the water snake clade. Second, branches to the outgroups are long relative to most of the basal internodes in the ingroup. To explore the effects of long outgroup branches on ingroup topology, we reanalyzed our data under the parsimony criterion with outgroup taxa excluded.

A consensus of the two most parsimonious trees that resulted from this analysis was largely congruent with

the consensus MPTs + outgroups with two important exceptions (Fig. 4). First, topology within the semifossorial clade changed to a fully symmetrical arrangement with *Storeria* as the sister group to *Clonophis* + *R. alleni* and *Virginia* as the sister group to *R. rigida* + *Seminatrix*. In addition, relationships at the base of the water snake clade collapsed to an unresolved trichotomy. Notably, we found very strong support for a bipartition between the semifossorial clade and the rest of the thamnophiines and moderate support for a bipartition between the watersnake group and the rest of the thamnophiines. Bootstrap support for these deeper nodes generally increased as third position transitions were downweighted, although support decreased at many shallow nodes when these transitions were excluded. Sensitivity of bootstrap support and topology within regions of the tree characterized by short internodes to the inclusion of outgroup taxa is consistent with the hypothesis of LBA (Huelsenbeck, 1997, 1998). Our ingroup-only analysis suggests that some of the differences between the parsimony and the likelihood estimates of relationship and of the bootstrap support for those relationships may be due to the effects of long branch attraction between the outgroups and certain ingroup taxa.

Phylogeny of Thamnophiine Snakes

A robust hypothesis of relationship for the thamnophiines is shown in Fig. 5. This topology is based on the ML topology with poorly supported nodes (<65% bootstrap support) collapsed. Relationships among the three major clades found in this study are unresolved, as are many of the within-clade relationships of the semifossorial group. However, strong to very strong support is found for relationships within the garter snakes and water snakes and for the unexpected relationship of *R. alleni*, *R. rigida*, and *Seminatrix pygaea*.

Our tree highlights many interesting patterns of evolution within this group and points to directions for future research. The three major clades recovered in this study correlate roughly with the ecology and diet of group members. Whereas the final determination of ancestral states will depend on the resolution of currently unresolved relationships within the semifossorial group and water snake group, certain dietary and ecological characters appear to be correlated with each major thamnophiine clade: terrestrial generalism in the garter snakes, semiaquatic piscivory in the water snakes, and semifossorial vermivory in the semifossorial group. This suggests that early thamnophiine differentiation proceeded along three distinct ecological trajectories. The ML phylogram suggests that these lineages diverged from each other at roughly the same time, deep in the history of the group.

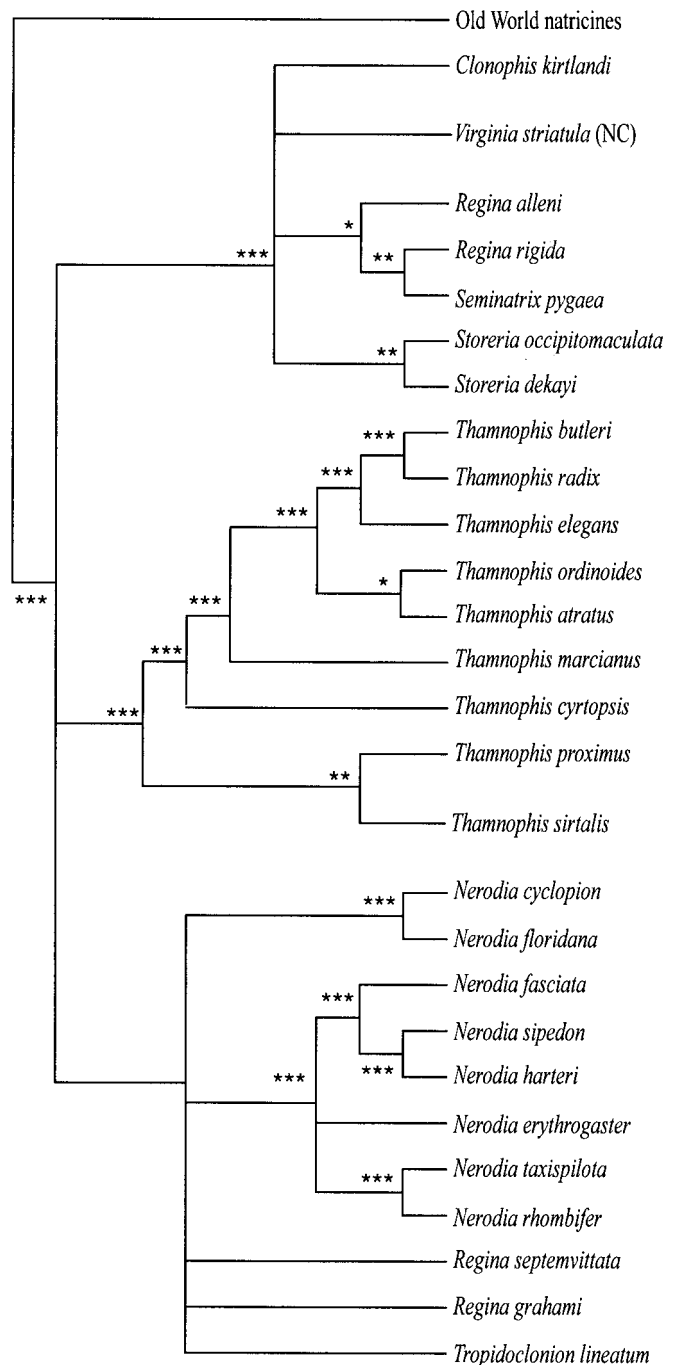


FIG. 5. Preferred phylogenetic hypothesis for thamnophiines. Tree shown is based on the maximum-likelihood tree (Fig. 6) with poorly supported nodes (bootstrap proportions <65%) collapsed. Our data resolve relationships at many levels of the tree and suggest that the thamnophiine radiation is composed of three main lineages. Relationships among these lineages are uncertain as are relationships at the base of the water snake and semifossorial clade. ***ML bootstrap support ≥ 95 ; **ML bootstrap support ≥ 80 ; *ML bootstrap support ≥ 70 .

Previous Hypotheses of Relationship

Our results conflict with earlier molecular and morphological studies of relationships within this group. A

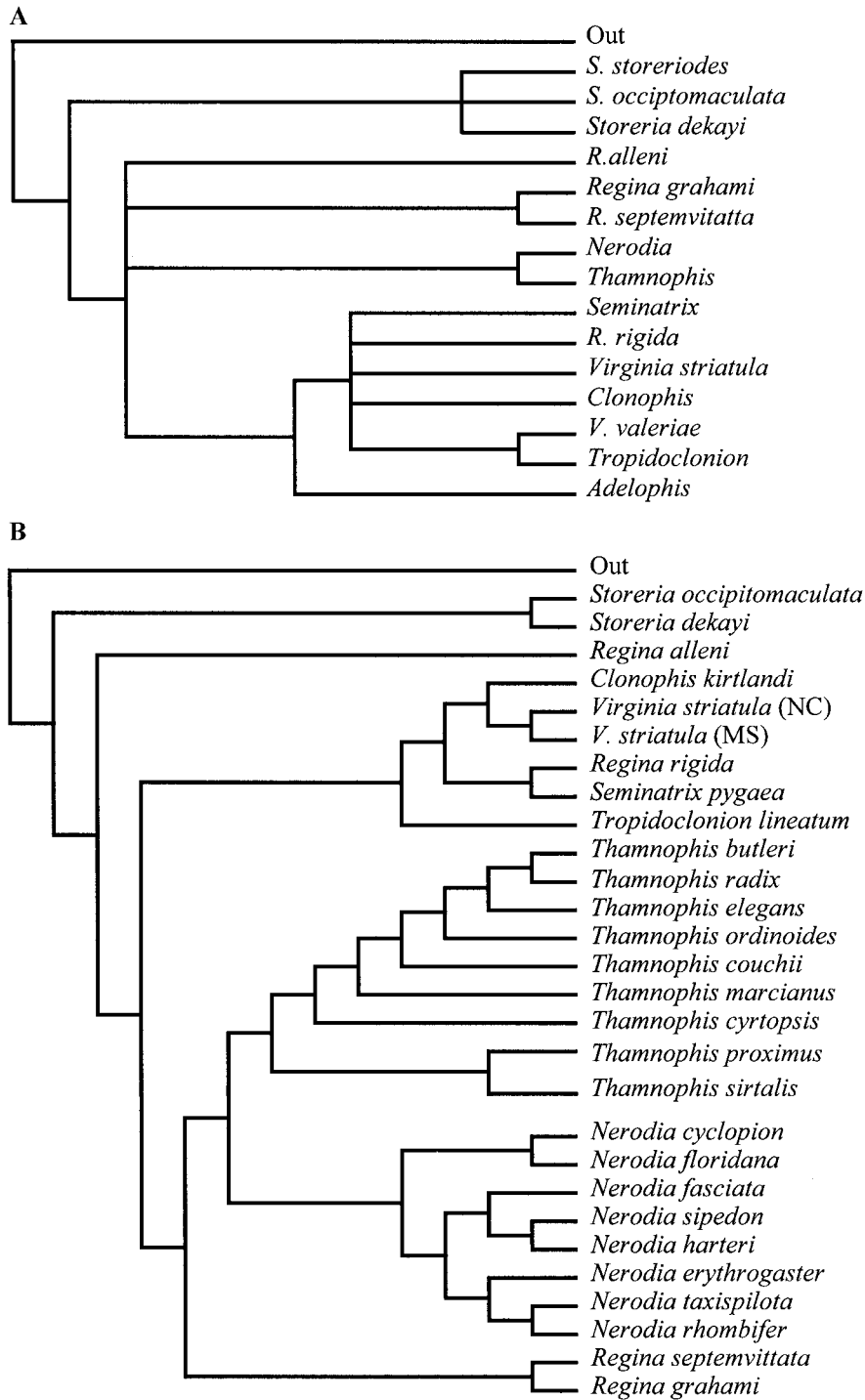


FIG. 6. Allozyme tree and allozyme constraint tree. (A) Phylogeny of the Thamnophiini based on allozyme data (Lawson, 1985). (B) Most parsimonious tree recovered under the topological constraint of the allozyme tree. Tree length = 4460 steps; Consistency Index = 0.352. The constrained tree is 42 steps longer than the unconstrained most parsimonious tree.

previous study using allozymes (Lawson, 1985), although unresolved at many levels of the phylogeny, is incongruent with the topologies recovered in our study (Fig. 6). In particular, Lawson (1985) found *Storeria* to be the most basal member of the tribe and *Tropidoclo-*

nion to be relatively derived and associated with many of the taxa in our semifossorial clade (Fig. 6). The allozyme tree, like our mitochondrial trees, suggests that *Regina* is polyphyletic with respect to other thamnophiines. Because of differences in taxon sampling, it

was not possible to easily combine the allozyme data with the mitochondrial data. To evaluate the degree to which our data supported a topology congruent with that of the allozyme tree, we performed a parsimony analysis using the allozyme topology as a backbone constraint. The single tree recovered is substantially longer than the unconstrained trees (Fig. 6), strongly suggesting that the DNA data are not congruent with the allozyme hypothesis.

Semifossorial Thamnophiines: A Natural Group

Within the semifossorial group, genetic differentiation was high. Intrageneric genetic distances ranged from 8 to 10% (uncorrected *p*) across all three genes and divergence was also high within *Storeria* (*p* = 8%). None of the six genera in this group were particularly closely related, suggesting that these species diverged from each other relatively early in the history of the tribe (Fig. 3). In contrast to an earlier study (Lawson, 1985) that found *Storeria* to be the most basal thamnophiine, our analyses suggest that this genus is more recently evolved, although its exact affinities could not be confidently determined. Topology in this clade varied with optimality criterion, weighting scheme, and inclusion or exclusion of outgroup taxa. This is partly due to the combination of short intermodes at the base of the semifossorial clade and long terminal branches.

Seminatrix as a Crayfish Snake?

The single best-supported intergeneric clade within the semifossorial group was also the most surprising: *R. alleni* + (*R. rigida*, *S. pygaea*). Although a close relationship has been posited previously for *R. alleni* and *R. rigida* on the basis of scale microornamentation (Price, 1983) and tooth morphology (Rossman, 1963, 1985; Price, 1983), there have been no previous suggestions of a sister group relationship between *R. rigida* and *S. pygaea*. Similarities in microornamentation pattern among *R. alleni*, *R. rigida*, and *S. pygaea* have previously been interpreted as the result of convergence upon a semiaquatic habit (see discussion in Lawson, 1985). However, if our hypothesis is correct, these characters should be regarded synapomorphies for a clade comprising these three species. Given the topologic instability of this region of the tree, this result should be interpreted cautiously. Future work should focus on sampling of additional semifossorial species including *Storeria storerioides* and *Virginia valeriae*, in an effort to break up long branches within this group (e.g., Graybeal, 1998)

Garter Snake Systematics

Although recent biochemical and molecular studies (de Queiroz and Lawson, 1994; Lawson, 1987; Boundy, 1999) have greatly enhanced our knowledge of garter snake phylogeny, relationships at nearly every level within this genus remain poorly resolved. The mitochon-

drial DNA characters sampled here appear to perform exceptionally well in resolving garter snake relationships, as shown by the high degree of resolution and bootstrap support for nodes in this clade. Although we have sampled less than one third of all the *Thamnophis* species, we are cautiously optimistic that future studies using these characters with additional taxon sampling will greatly improve our understanding of relationships within this group.

In agreement with earlier studies of protein electrophoretic data (Lawson, 1985, 1987), our results strongly support the monophyly of *Thamnophis*. We also found strong support for a *T. elegans*/*T. butleri*/*T. radix* complex which had been suggested in an earlier study using combined Cytb and allozyme data (de Queiroz and Lawson, 1994). In addition, we found the sequences of *T. butleri* and *T. radix* to be nearly identical to each other (5-bp differences over all sequence examined). It has been suggested that the Wisconsin population of *T. butleri*, which is disjunct from the Indiana–Michigan–Ohio distribution of the species, hybridizes with *T. radix* (Rossman *et al.*, 1996). The *T. butleri* individual sampled for this study came from the Wisconsin population and thus the low genetic difference between it and *T. radix* might be a consequence of hybridization.

Our analyses also revealed a major split between *T. sirtalis* and *T. proximus* (hereafter called the *sirtalis* group) and the rest of the garter snakes sampled (hereafter called the *elegans* group). This division is strongly supported by bootstrapping and is congruent with de Queiroz and Lawson's (1994) consensus topology. *T. sirtalis* and *T. proximus* were nearly as divergent from each other (*p* > 7%) as either was to members of the *elegans* group (average *p* = 7.3%). Distances within the *elegans* group were lower (*p* = 3–5%), suggesting that this clade has undergone a more recent radiation subsequent to its divergence from the *sirtalis* group.

Water Snake Systematics

Lawson (1987) recognized three distinct lineages of *Nerodia*, although the relationships among these lineages were not resolved. We recognize two clades of *Nerodia*: a *sipedon* group that encompasses Lawson's *sipedon* and *taxispilota* lineages (*N. sipedon*, *N. harteri*, *N. fasciata*, *N. erythrogaster*, *N. taxispilota*, and *N. rhombifer*) and a *cyclopion* group that is identical to Lawson's (*N. cyclopion*, *N. floridana*). Within the *sipedon* clade, only the position of *N. erythrogaster* is poorly supported. Under MP, placement of it as sister to *N. taxispilota* + *N. rhombifer* is equally parsimonious with its position as the sister group to *N. fasciata* + (*N. sipedon* + *N. harteri*). Under maximum-likelihood, this latter topology is preferred, although it receives poor bootstrap support.

In both MP and ML analyses, *Nerodia* is paraphyletic with respect to *R. grahami*, *R. septemvittata*, and

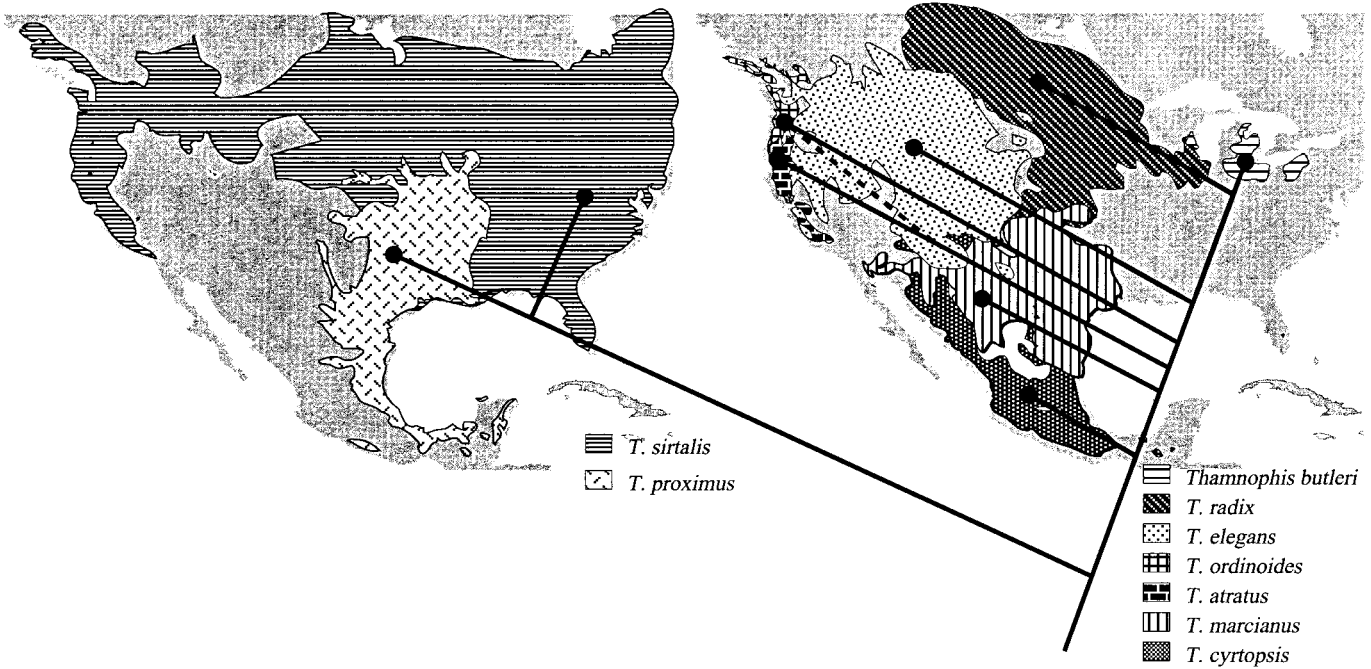


FIG. 7. Biogeography of *Thamnophis*. Ranges shown are approximate and taken from Rossman *et al.* (1996). The basal members of one main lineage of garter snakes have a Mexican and southern U.S. distribution, whereas the more distal species in the tree are found northward and westward. This supports the hypothesis that at least one major lineage of garter snakes originated in Mexico. The biogeographic pattern of the lineage containing *T. sirtalis* is consistent with the hypothesis of a second invasion of North America, although taxon sampling is too sparse to generate high confidence for this scenario.

T. lineatum. However, the short internodes connecting the two *Nerodia* lineages with *Tropidoclonion* and the two *Regina* species and the lack of bootstrap support for these deeper nodes suggest that this topology is not particularly stable. The novel grouping of *R. grahami* with *Tropidoclonion* is likewise poorly supported, although both parsimony and likelihood analyses recover this relationship. Although this result is surprising, there are some possible morphological synapomorphies that unite these taxa, including lateral stripes, dark spots along the midline of the belly, and dorsal striping in *Tropidoclonion* and some *R. grahami* (A. de Queiroz, pers comm.).

With the exception of *Tropidoclonion*, all of the members in the water snake group are semiaquatic, specialize on aquatic prey (fish and amphibians, or in the case of *R. grahami* and *R. septemvittata*, crayfish), and are restricted in range to eastern and southern North America. *Tropidoclonion* prefers drier habitats, feeds on earthworms, and is distributed more centrally and westerly than the other taxa in the group. Our results suggest that this taxon represents a secondarily terrestrial water snake or crayfish snake. If true, this would suggest that, in addition to repeatedly invading aquatic habitats (e.g., Drummond, 1980; Schaeffel and de Queiroz, 1990; de Queiroz, 1992), thamnophiines have also reinvaded terrestrial niches over the course of their evolution.

Polyphyly of *Regina*

Almost since its erection, the composition of the genus *Regina* has been controversial. Scale coloration, hemipenial morphology, osteology (Rossman, 1963, 1985), visceral morphometrics (Rossman *et al.*, 1982), and cranial myology (Varkey, 1979) have been used to support *Regina* monophyly. However, microornamentation characters (Price, 1983) and, more recently, allozymes (Lawson, 1985) suggest that there are two to three evolutionarily independent lineages subsumed in the genus. Our results reveal a major split among the four species of *Regina*: *R. grahami* and *R. septemvittata* are closely allied with the *Nerodia* group and *R. alleni* and *R. rigida* are related to the semifossorial group. Furthermore, our best ML and MP trees suggest that no pairs of *Regina* species are sister taxa! Although our analysis failed to robustly determine *Regina* relationships within the tribe (due to the relatively short internodes at the base of the *Nerodia* group), parametric and nonparametric tests reject *Regina* monophyly (M. E. Alfaro, in prep). Our results suggest that a reevaluation of the taxonomic status of *Regina* is warranted.

Biogeography of the Garter Snakes and Water Snakes

There have been relatively few studies focused on the phylogeography of *Thamnophis*. Ruthven (1908) suggested that Mexico was the center of origin for the

garter snakes, although this hypothesis was based largely on early and questionable ideas about biogeography, such as that species near the center of the distribution represent the largest and most differentiated forms of a complex. Additional evidence for a Mexican origin of this group comes from de Quieroz and Lawson (1994) who found *T. fulvus*, a Mexican species, to be the sister group to all other *Thamnophis*. Garter snake diversity is highest in Mexico, with 18 of 30 or so described species occurring there. In our study, the biogeographic pattern displayed by the species in both the *elegans* group and the *sirtalis* group supports a modified version of Ruthven's (1908) hypothesis (Fig. 7). The most basal members of the *elegans* group (*T. cyrtopsis* and *T. marcianus*) have a southwestern distribution. More distal taxa on the tree are distributed in the northwest and the most recent species in this clade of garter snakes (*T. radix* and *T. butleri*) are also the most eastern. The pattern of diversification within the clade including *T. proximus* and *T. sirtalis* is unclear due to the poor taxonomic sampling of this portion of the garter snake tree. The present data suggest that at least one and possibly two lineages of *Thamnophis* have colonized northern North America from central or northern Mexico.

The lack of strongly supported relationships at the base of the water snake group makes inferences about biogeography in this clade problematic. However, tentative hypotheses may be made in regard to the distribution of *Nerodia*. The most basal members, *N. cyclopion* and *N. floridana*, are restricted to the Florida panhandle and the gulf coast, with *N. cyclopion* also found in the Mississippi Valley. Taxa in the second major lineage of *Nerodia* are, in general, much more broadly distributed throughout the eastern and southern United States. Our data are consistent with Lawson's (1987) hypothesis that Pleistocene glaciation drove speciation in *N. cyclopion* and *N. floridana* by dividing the ancestral population into eastern and western refugia. We suggest that the more northerly ranges of *N. erythrogaster* and *N. fasciata* represent a post-Pleistocene expansion of these taxa from gulf coast refugia. We hypothesize that the extreme northern and eastern range of *N. sipedon*, and its absence from the gulf coast region, results from a relatively recent splitting from *N. fasciata* and subsequent northerly invasion. We also suggest that *N. harteri*, with its extremely disjunct distribution in stream systems of central Texas, speciated as the result of an easterly range contraction of *N. sipedon*, its sister taxon.

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