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Coalescent Species Delimitation in Milksnakes (Genus *Lampropeltis*) and Impacts on Phylogenetic Comparative Analyses

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Abstract.—Both gene-tree discordance and unrecognized diversity are sources of error for accurate estimation of species trees, and can affect downstream diversification analyses by obscuring the correct number of nodes, their density, and the lengths of the branches subtending them. Although the theoretical impact of gene-tree discordance on evolutionary analyses has been examined previously, the effect of unsampled and cryptic diversity has not. Here, we examine how delimitation of previously unrecognized diversity in the milksnake (*Lampropeltis triangulum*) and use of a species-tree approach affects both estimation of the *Lampropeltis* phylogeny and comparative analyses with respect to the timing of diversification. Coalescent species delimitation indicates that *L. triangulum* is not monophyletic and that there are multiple species of milksnake, which increases the known species diversity in the genus *Lampropeltis* by 40%. Both genealogical and temporal discordance occurs between gene trees and the species tree, with evidence that mitochondrial DNA (mtDNA) introgression is a main factor. This discordance is further manifested in the preferred models of diversification, where the concatenated gene tree strongly supports an early burst of speciation during the Miocene, in contrast to species-tree estimates where diversification follows a birth–death model and speciation occurs mostly in the Pliocene and Pleistocene. This study highlights the crucial interaction among coalescent-based phylogeography and species delimitation, systematics, and species diversification analyses. [Divergence-time estimation; diversification rates; Lampropeltini; gene-tree/species-tree discordance; mtDNA introgression; Pleistocene diversification.]

The description, diagnosis, and delimitation of species is essential across all fields of biology and is necessary for studies conducted within a phylogenetic framework, as missing taxa are known to decrease accuracy in inferring taxonomic relationships, estimating models of evolution, and calculating divergence times (Hillis 1996, 1998; Graybeal 1998; Poe 1998; Pollock and Bruno 2000; Pollock et al. 2002; Zwickl and Hillis 2002; Heath et al. 2008; Nabhan and Sarkar 2012; Smith et al. 2013). Species may be missing from phylogenies because samples from known species were not available or included by researchers, or due to unrecognized diversity. With multi-locus data surveyed across the group of interest, it is now possible to determine extant species diversity in a group prior to phylogenetic analyses, using coalescent-based models of species delimitation (Leaché and Fujita 2010; Burbrink et al. 2011; Camargo et al. 2012; Spinks et al. 2012; Smith et al. 2013).

For many groups, new species are still being discovered using traditional morphological data and techniques (e.g., frogs, Ávila et al. 2011; annelids, Rota 2013) or using molecular data in a phylogeographic context (e.g., vipers, Stümpel and Joger 2009; tree shrews, Roberts et al. 2011; gobies, Vanhove et al. 2012; scorpions, Bryson et al. 2013). However, species boundaries are not always explicitly tested using multi-locus data, despite evidence that suggests reliance on morphological characters or patterns from single-locus/gene-tree analyses can potentially mislead phylogenetic inferences (Burbrink et al. 2000; Bossu and Near 2009; Smith et al. 2012). Ultimately, this can result in species diversity remaining undescribed and unsampled (Leaché and Fujita 2010; Smith et al. 2012).

One major problem associated with unsampled species in phylogenetic reconstruction is the impact of missing taxa on comparative analyses. Incomplete sampling may result in an increase in the overall divergence-time estimates due to an oversampling of deep nodes (Cusimano and Renner 2010). Measures that quantify diversification will then incorrectly indicate that rates of speciation have decreased toward the present (Pybus and Harvey 2000; Rabosky and Lovette 2008; Cusimano and Renner 2010; Smith et al. 2013). The use of gene trees also adds to the problem of divergence-time overestimation (McCormack et al. 2010; Smith et al. 2013). Because gene divergences predate species divergences, gene-tree methods will incorrectly yield estimates of older branching times relative to species divergences (Edwards and Beerli 2000; Carstens and Knowles 2007; Burbrink and Pyron 2011). This has been demonstrated empirically in *Aphelocoma* jays, where the dates generated using concatenated data sets are older than those from multi-locus species trees (McCormack et al. 2010).

For taxa used in studies examining the timing and tempo of speciation, a basic understanding of species numbers and species-tree phylogeny is thus crucial. A key example is the milksnake (*Lampropeltis triangulum*), one of the most well-known New World snakes owing to its vivid red, black, and yellow tri-color patterns.

Despite its use in comparative phylogenetic studies (Pyron and Burbrink 2009a; Burbrink and Pyron 2010; Burbrink et al. 2012) and role as a classic example of Batesian mimicry (Brattstrom 1955; Grobman 1978; Greene and McDiarmid 1981; Brodie 1993; Brodie and Janzen 1995; Pyron and Burbrink 2009a), it has never been examined using a coalescent framework. The mimicry of milksnakes to the venomous coralsnakes even inspires the common mnemonic "Red on yellow, kill a fellow; Red on black, friend of Jack," used to discriminate between the two based on their color patterns. The 25 currently recognized subspecies of milksnake (following Williams 1988) range from southeastern Canada, across the United States to the Rocky Mountains, and south to Ecuador, making the inter-continental distribution of the milksnake one of the largest of any squamate (Williams 1988). It is found in a variety of habitats, including temperate deciduous forest, long-leaf pine woods, grasslands and prairies, desert, and sub-tropical to tropical habitat, and displays an astounding variety of color patterns, body sizes, and diets across this range (Fitch and Fleet 1970; Dyrkacz 1977; Brown 1979; Williams 1988; Palmer and Braswell 1995; Rodríguez and Drummond 2000; Pyron and Burbrink 2009a). These dramatic ecological and phenotypic differences, consistent with predictions of the general lineage species concept (followed here; de Queiroz 1998, 2007), suggest that *L. triangulum* may comprise multiple unrecognized species.

Recent molecular studies within the tribe Lampropeltini, which include many commonly known snakes such as kingsnakes (e.g., *L. getula*, *L. mexicana*, and *L. pyromelana*), ratsnakes (e.g., *Pantherophis obsoletus, P. gutattus*), and pinesnakes (e.g., *Pituophis melanoleucus*), have found that numerous taxa within this group historically classified as single species probably consist of multiple distinct lineages (Rodríguez-Robles et al. 1999; Burbrink et al. 2000; Rodríguez-Robles and De Jesús-Escobar 2000; Burbrink 2002; Bryson et al. 2007, 2011; Pyron and Burbrink 2009b, 2009c). Within *Lampropeltis* alone, two distinct species have recently been found to contain cryptic taxa, resulting in the recognition of seven rather than two species (*L. getula* complex, Pyron and Burbrink 2009b, 2009c; *L. pyromelana* complex, Burbrink et al. 2011).

Several of these previous studies also indicate that the milksnake may not form a monophyletic group with respect to other species of *Lampropeltis*; gene trees reconstructed from mitochondrial DNA (mtDNA) and single-copy nuclear DNA markers (scnDNA) result in differing topological placement of milksnake within *Lampropeltis* (Bryson et al. 2007; Harper and Pfennig 2008; Pyron and Burbrink 2009d). These studies further suggest that there may be multiple distinct lineages within *L. triangulum*, possibly including *L. alterna*, the gray-banded kingsnake. However, none of these studies focused on the milksnake specifically, all included a limited number and distribution of samples, and all relied on gene trees rather than species trees.

Diversification analyses of Lampropeltini have found that, unlike many other North American taxa (Rand 1948; Avise 2000; Johnson and Cicero 2004), speciation in this tribe occurred mostly during the Miocene and Pliocene and slowed toward the present, with few species originating during the Pleistocene (Pyron and Burbrink 2009d; Burbrink and Pyron 2010). However, these studies did not include the most recently elevated species within *Lampropeltis* and relied on the topology and branch lengths from concatenated gene trees. The combination of unsampled taxa and gene-tree based approaches may be especially problematic for prior analyses that included *Lampropeltis* and relied potentially on inaccurate divergence times and branch lengths.

Here, we address the following questions: 1) Is the milksnake a single, wide-ranging species or multiple, independently evolving species?, 2) Is the milksnake monophyletic within *Lampropeltis*?, and 3) Do different approaches (i.e., gene trees vs. species trees) alter results with respect to taxonomy, timing, and tempo of diversification for *Lampropeltis*? To examine species diversity and timing of diversification in *Lampropeltis*, we use data collected from 11 nuclear loci and individuals spanning the geographic range of *L. triangulum* to perform Bayesian species delimitation. We also include representatives from all species in *Lampropeltis* to then infer a species tree for the genus. We delimit species using coalescent methods based on guide trees from both a mitochondrial gene tree and from a population structure analysis. Results from our study demonstrate the need to explicitly delimit taxa for species-tree analyses, and underscores the importance of including all species to properly understand the tempo of species diversification and other downstream comparative analyses.

Materials and Methods

Data Collection

We obtained tissues from 276 milksnakes ranging across North America, Central America (CA), and South America (SA) (Fig. 1), including 21 of the 25 subspecies. We also included 49 samples of the remaining 14 species in the genus *Lampropeltis* ($\geq$2 individuals for each species, following taxonomy in Pyron and Burbrink 2009b, 2009c; Burbrink et al. 2011, and including *L. webbi*, Bryson et al. 2005) and 2 samples each for the outgroup taxa, *Arizona elegans* and *Cemophora coccinea* (Pyron and Burbrink 2009d; online Appendix 1 http://dx.doi.org/10.5061/dryad.420h7). The four subspecies of milksnake not included in our study (*L. t. andesiana*, *L. t. blanchardi*, *L. t. campbelli*, and *L. t. oligozona*) have fairly small geographic distributions (Williams 1988), and so presumably their omission will have minimal impact on our results. We also note that *L. t. elapsoides*, treated here as a subspecies prior to species delimitation analyses for clarity, has been considered a full species in previous work (Pyron and Burbrink 2009a, 2009d).

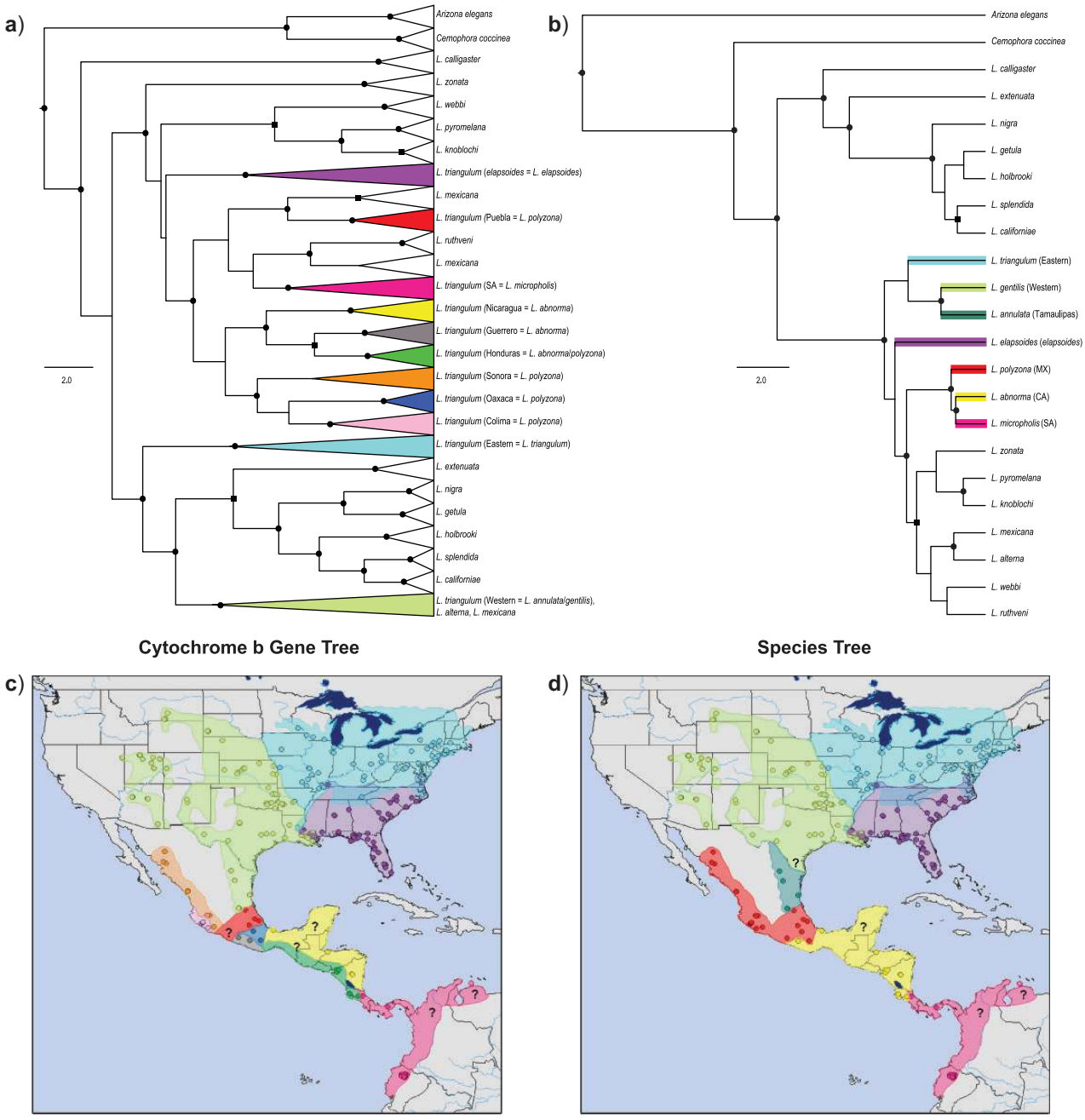

FIGURE 1. Cytochrome *b* (Cytb) gene tree a) and multilocus species tree b) for *Lampropeltis* and outgroups (Cytb tree, $n = 329$; species tree, $n = 124$) inferred using Bayesian inference in the program BEAST/*BEAST. All species/clades include ≥2 individuals (see online Appendix 1 for specimen details). For (a), Cytb milksnake clades are indicated by colored lineages, followed by the clade name in parentheses and the corresponding delimited species name; names and colors correspond with the Cytb-lineage map c). For the species tree (b), the delimited milksnake species are indicated by colored branches, with the lineage name in parentheses; names and colors correspond with the species-tree map d). Where applicable, colors and names are conserved between the Cytb and species trees, although it should be noted that there are fewer delimitable species than there are Cytb clades for milksnakes, as some Cytb clades are collapsed within the species delimitation. Posterior probabilities ≥95% indicated by filled circles; ≥85% and <95% indicated by filled squares. Note that all *L. alterna* ($n = 13$) and one *L. mexicana* are included within the Western *L. triangulum* clade in the Cytb tree.

DNA was extracted using Qiagen DNeasy kits (tissue protocol) from samples of shed skin, liver, muscle tissue, or whole blood. Of the 329 total samples, 23 individuals were sequenced previously for between 1 and 3 loci; these individuals and their corresponding GenBank numbers are included as online Appendix 2 (http://dx.doi.org/10.5061/dryad.420h7). We optimized the amplification and sequencing protocols for 1 mtDNA gene (cytochrome *b*; Cyt*b*) and 11 scnDNAs, including 5 anonymous loci developed using

TABLE 1. Loci Amplified for *Lampropeltis*

Locus	Length (bp)	Model	Variable sites *Lampropeltis*	Variable sites milksnakes	Number of sequences
Cytb	1,117	TPM + Γ + I	448	341	329
NT3	481	GTR + Γ	30	26	105
PRLR	552	TRN + Γ + I	30	21	115
GAD2 intron 15	541	HKY + Γ + I	36	24	111
NAV intron 5	561	HKY + Γ	30	22	115
SPTBN1 intron 1	839	TPM + Γ + I	59	44	124
VIM introns 5	584	HKY + I	43	25	118
CL4	373	HKY + Γ + I	25	17	102
LAT clone	705	TRN + Γ	35	25	100
2CL3	429	F81 + Γ	20	17	106
2CL4	376	TRN + Γ	25	15	103
2CL8	466	HKY + Γ + I	69	56	120

Note: The length in base pairs, the model of evolution, number of variable sites, and total number of sequences for each locus are listed. Additional details for each locus and PCR protocols are listed in Appendix 2.

the protocol from Noonan and Yoder (2009). Details for loci, primers, and polymerase chain reaction (PCR) protocols are listed in Appendix 1.

For Cyt*b*, we sequenced all individuals, and for the scnDNA loci, we sequenced a subset of individuals (n = 124), which were chosen to represent a wide geographic range within the United States, and included all samples from Mexico, CA, and SA, as well as the representatives from all *Lampropeltis* species (online Appendix 1). All sequences were generated using Sanger sequencing and were aligned by eye using SEQUENCHER 4.5 (Genecodes 2000). No gaps were found in any of the protein-coding genes. The phase of heterozygous genotypes was resolved using PHASE v.2.1.1 (Stephens and Donnelly 2003) and the most probable pair of alleles was used for each heterozygous individual. For all genes and subsequent analyses, we determined the most appropriate substitution model using Bayesian Information Criterion in the program jModeltest (Table 1; Posada 2008).

Species Delimitation

To delimit species, we used Bayesian Phylogenetics and Evolution (BPP; Yang and Rannala 2010), a genealogical method that uses multiple independent loci in a coalescent framework using a reversible-jump Markov chain Monte Carlo (rjMCMC) method to estimate Θ_a (effective population $N_e \times$ mutation rate μ for each species), τ_a (the time of origin for each species), and τ_d (the timing of diversification into two descendent species). Results in the form of posterior probability distributions (PP) indicate whether two or more predefined lineages can be differentiated from each other accounting for coalescent uncertainty. This method has previously been utilized for delimiting a number of squamate species (Leaché and Fujita 2010; Burbrink et al. 2011; Cox et al. 2012) and, when using multiple individuals and loci, has been found to be robust in simulation studies when migration levels are low ($\leq\sim$1 migrant per generation; Zhang et al. 2011).

Accurate results for BPP rely on a user-specified phylogenetically meaningful guide tree (Leaché and Fujita 2010) that depicts the proposed species and their topological relationships; guide trees may be based on data from various sources of evidence, including gene trees, population structure analyses, subspecies designations, ecology, or morphology. To test species hypotheses for the milksnake, we constructed guide trees in two ways. First, with both the mtDNA and scnDNA data set (the mtDNA data set was reduced to the same individuals used in the scnDNA data set for all subsequent analyses, see online Appendix 1), we used the program Structurama (Huelsenbeck and Andolfatto 2007) to infer groups and assign individuals to these groups. Structurama uses a Dirichlet process prior for a random number of k populations with the initial concentration parameter of populations set to 5 to estimate the number of groups and assignment of individuals with the highest probability. Using MCMC, we ran Structurama four times for 1×10^6 generations, taking every 100th sample, with the first 25% of samples discarded as burnin. The appropriate value of k was chosen as the one with the highest posterior probability value. To generate the guide tree, the resulting populations were then treated as terminal taxa in *BEAST (Drummond et al. 2012), with the resulting species tree used as a guide for the BPP analyses. We then ran *BEAST using both the mtDNA + scnDNA data set and the scnDNA data set alone; this resulted in identical topologies and, hence, a single guide tree.

Second, we inferred a gene tree for Cytb using all samples as the basis for a second guide tree. The mtDNA gene tree was inferred in BEAST v.1.7.2, chains were run for 5×10^7 generations, and sampled every 1000th generation. The resulting lineages from the mtDNA tree were treated as terminal taxa and run in *BEAST using the scnDNA data set, with the resulting tree used as the guide tree in BPP analyses. All *BEAST analyses conducted to generate guide trees were run for 1.5×10^6 generations, sampled every 1000th generation, with a burnin of 25%.

For all guide trees, BPP was run using the algorithm 0, and we adjusted the fine-tuning parameters to ensure swapping rates ranged between 0.30 and 0.70 for each parameter, allowing the rjMCMC to mix properly among species-delimitation models. Zhang et al. (2011) demonstrated that the performance of BPP may be sensitive to the prior distributions of ancestral population size (Θ) and root age (τ_o). Similar to previous studies (Leaché and Fujita 2010; Burbrink et al. 2011; Cox et al. 2012; Smith et al. 2012), we parameterized both Θ and τ_o using a gamma (Γ) distribution (α, β) for the following: large populations and deep divergences ($\alpha = 1$, $\beta = 10$); small ancestral populations and shallow divergences ($\alpha = 2$, $\beta = 2000$); and large ancestral populations ($\alpha = 1$, $\beta = 10$) with shallow divergences ($\alpha = 2$, $\beta = 2000$). A large ancestral population with shallow divergences is considered the most conservative model with respect to favoring speciation events (Leaché and Fujita 2010). For each of these, we ran a minimum of three analyses using different starting seeds for 5×10^5 generations with a burnin of 1.5×10^4, and thinning every five generations. We ran all of these analyses using the two different guide trees. Following Burbrink et al. (2011), we also randomized the individuals in each clade to ensure that BPP was not able to arbitrarily delimit randomized (i.e., incorrect) groups.

Migration Rates

Species-delimitation programs such as BPP (Yang and Rannala 2010) only assess error due to incomplete lineage sorting, meaning migration between lineages may violate assumptions (Zhang et al. 2011). Assessing gene-flow, or a lack thereof, can also provide support as to whether lineages should be treated as independent species. As such, we examined rates of migration to develop a better understanding of how much gene flow occurs among the resulting geographically adjacent milksnake species using Migrate-n v.3.2.16 (Beerli 2008). We also included *L. alterna* in these analyses to better understand whether there is gene flow between that taxon and any newly delimited species of milksnake (Bryson et al. 2007). Migrate-n was run using the complete scnDNA data set for 1×10^4 generations with four Markov chains, sampled every 100th generation, and with the first 25% of samples discarded as burnin. A mean generation time for *Lampropeltis* of 2.5 years was used for analyses (Werler and Dixon 2000; Ernst and Ernst 2003). Each run was repeated four times with different starting seeds to ensure consistency between runs.

We also constructed a combined scnDNA network for resulting lineages using Splitstree v.4.12.3 (Huson and Bryant 2006) with the Neighbor-net algorithm. Networks can further help visualize reticulate relationships among taxa and possible mixed ancestry or hybrid individuals. We used 1000 nonparametric bootstrap replicates to assess support for the resulting network groups and tested for recombination using the φ statistic in Splitstree.

Species-Tree Estimation

To estimate a species tree for those taxa that were delimited by BPP with high support values under all parameterizations of Θ and τ_o, we used *BEAST (Drummond et al. 2012) implemented in BEAST v.1.7.2. This method uses a multispecies coalescent model to estimate the species tree from multiple genes and multiple individuals per species, while taking into account incomplete lineage sorting. In addition, *BEAST uses a relaxed clock model (Drummond et al. 2006, 2012) to estimate divergence times for the species tree. For this analysis, we used the scnDNA data set consisting of the milksnake lineages recovered in the BPP analyses and all other currently recognized species in the genus *Lampropeltis*. The locus 2CL3 was not used in the *BEAST analysis because it did not amplify for all species. Because of the possibility of mtDNA introgression (discussed later), which violates assumptions of no admixture, we ran the species-tree analysis without the Cytb data. Individuals were assigned to species based on the results from the BPP analyses.

For the species tree and divergence-time estimation, we used an uncorrelated lognormal tree prior with a Yule speciation-process prior and calibrated the tree using two appropriate fossil constraints. From Holman (2000), the two calibrations are as follows: 1) The divergence time between the genera *Lampropeltis* and *Cemophora* was given a mean date of 13.75 Ma (95% Highest Posterior Density [HPD]: 8.4–24.4 Ma) based on the oldest known *Lampropeltis*, *L. similis*, from the medial Barstovian of the Miocene; 2) The divergence time between the *L. getula* complex and *L. extenuata* was given a mean date of 6.8 Ma (95% HPD: 4.75–9.94 Ma) based on the oldest known fossils of *L. getula* and *Stilosoma* (=*Lampropeltis*) *venustum* from the middle Hemphillian of the Miocene. Following Pyron and Burbrink 2009d, we enforced these soft constraints using lognormal distributions, with the age of the fossil as the mean time for the divergence at the node and a 95% prior distribution around the mean as soft bounds for the timing of bifurcation at the stem of that node. We ran *BEAST for 200–300 million generations, sampled every 5000 generations, and assessed stationarity using Tracer v.1.5 (Drummond and Rambaut 2007). The analysis was run four times to ensure consistency among results.

Concatenated scnDNA Tree

To explore the difference in divergence times between gene trees and species trees, we ran BEAST using the concatenated scnDNA data set with one representative for the same terminal taxa used in the species-tree estimation. Calibrations were the same as those used for the original species tree and we ran the concatenated data set for 5×10^7 generations, sampling every 1000th generation. We then calculated the Robinson–Foulds distance (Robinson and Foulds 1981) between the species tree and concatenated tree using R in the package

PHANGORN (Schliep 2011); this metric measures the number of bipartitions found in one tree but not the other, indicating the amount of topological discordance between trees.

Introgression

Results presented here (discussed later) and in a previous study (Bryson et al. 2007) have indicated possible mtDNA introgression within *Lampropeltis*, specifically with respect to *L. alterna* and *L. triangulum* from the western United States and northeastern Mexico. To determine whether introgression has occurred within *Lampropeltis*, we used the program JML v.1.01 (Joly 2012) to detect introgressed sequences. JML tests whether the minimum genetic distance between the sequences of two species is smaller than expected under a scenario that does not account for hybridization (i.e., incomplete lineage sorting) and uses the original sequence data for the locus tested, and the posterior distribution of species trees from *BEAST.

JML simulates new data for the locus of interest using the information contained in the species trees (e.g., topology, population sizes, and branch lengths) as well as locus-specific information taken from the *BEAST analysis, relative to the other loci used to infer the species tree (e.g., heredity scalar, mutation rate, nucleotide model parameters). The program then compares the minimum pairwise sequence distance between the species for all simulated data sets with the original data. If the observed values are smaller than 95% of the simulated data, it can be concluded that the model used by *BEAST (which assumes incomplete linage sorting and not hybridization) does not fit the data well for that locus, and that there has likely been introgression. The output files from JML indicate which, if any, species show introgression. To conduct this analysis, we inferred a species tree in *BEAST using the nuclear data set as well as Cytb to obtain relative rates for all loci; *BEAST was run for 200 million generations, sampled every 10,000 generations, and again assessed burnin using Tracer v.1.5 (Drummond and Rambaut 2007). We used the output from this *BEAST analysis in JML to determine whether introgression could be detected for any particular species and locus at $\alpha = 0.05$.

To further examine the likelihood of introgression within *Lampropeltis*, we ran an additional test for any species found to show mtDNA introgression in the JML analysis. Using code from Rabosky et al. (2009) in R v2.13.1 (R Development Core Team 2006), we tested whether observed gene trees for the potentially introgressed taxa were more likely due to incomplete lineage sorting or introgression. This method uses simulation to calculate the probability that discordance between a non-monophyletic mtDNA gene tree and monophyletic nuclear gene trees is due to incomplete lineage sorting.

Following the methodology outlined by Rabosky et al. (2009), we used RAxML v.7.0.4 (Stamatakis 2006) to construct maximum likelihood gene trees for the introgressed species using the GTRMIX model for each locus with 1000 bootstrap replicates under the rapid bootstrap algorithm. This approach conducts a bootstrap analysis and searches for the best maximum likelihood tree, resulting in a full maximum likelihood analysis in a single run. We determined the number of reciprocally monophyletic gene trees for the taxa being examined from the RAxML trees and then calculated the number of alleles/haplotypes found for each species and locus showing monophyly using DnaSP v.5 (Librado and Rozas 2009).

Using the observed number of alleles and waiting times scaled by relative N_e for the loci (Cytb = 0.25, scnDNA = 1.0), we simulated the joint distributions of waiting times to the most recent common ancestor for Cytb and the nuclear loci showing monophyly. We simulated 50,000 sets of waiting times, and determined the number of simulations where the time to the most recent common ancestor for mtDNA from species of interest exceeded the nuclear locus combinations (Rabosky et al. 2009). Results indicate the probability of obtaining a non-monophyletic mtDNA gene tree and monophyletic nuclear gene trees due to incomplete lineage sorting, based on the estimated coalescence times of a non-monophyletic mtDNA locus and the monophyletic nuclear loci (Rabosky et al. 2009).

Timing and Models of Species Diversification

We used the Wilcoxon signed-rank test in R to determine whether the concatenated tree results in significantly different mean divergence times when compared with the species tree. Following Burbrink and Pyron (2011), we calculated the scaled branching-time error by taking the difference between the mean gene-tree branching times and the mean species-tree branching times, divided by the depth of the corresponding nodes of the species tree. We regressed the scaled branching-time error against the branching times of the species tree in R. A significantly negative slope would indicate branching-time error decreases as nodes get older. To compare the temporal discordance of the concatenated tree and the species tree, we ran Pybus and Harvey's γ in the R package LASER (Rabosky 2007), which tests for early, late, or constant diversification (Pybus and Harvey 2000).

To further examine how the use of gene trees versus species trees affects downstream analyses, we also tested preferred models of species diversification for the concatenated tree and species tree. Using the function Misfits (available from FTB) and the packages APE (Paradis et al. 2004) and LASER (Rabosky 2007) in R, we compared the nine approximate likelihood coalescent models described in Morlon et al. (2010); these models include both time-constant or time-variable rates of speciation (λ) and extinction (μ) and time-constant or time-variable diversity.

We also assessed full likelihood standard models that had constant rates of speciation (Yule, Birth–Death [BD]),

variable rates of speciation due to diversity dependence (DDL, DDX), variable rates of speciation due to non-density dependent factors (SPVAR, Yule2, Monotonic Decay, and Hyberbolic Decay), variable extinction rates (EXVAR), or both variable speciation and extinction rates (BOTHVAR). Because the standard density dependent models do not account for extinction, we also tested maximum likelihood models that include extinction as an additional parameter for Diversity Dependence (DD + E) against BD, and Shift-Point (which allows for a shift in the parameters at a time point and is equivalent to Yule2 with extinction and carrying capacities) in the R package DDD (Etienne et al. 2012). The best-fitting model for each of the standard models, the coalescent models, and the models with extinction was determined by calculating corrected AIC values for small sample sizes (AICc; Burnham and Anderson 2002).

Results

Loci

Cytochrome *b* (1117 bp) was sequenced for 329 individuals (full or partial coverage; 448 variable sites for *Lampropeltis* and 341/1117 variable sites for all samples initially considered *L. triangulum*). The scnDNA loci (5908 bp total) for the subset of 124 samples resulted in 100–124 sequences for each gene, with a maximum of 69 and a minimum of 20 variable sites for *Lampropeltis* and a maximum of 56 and a minimum of 15 variable sites for samples initially considered as *L. triangulum* (Table 1; Appendix 1; online Appendix 1). Overall, only ~10% of the data was missing for all 12 loci combined. All newly generated sequences are deposited in GenBank (Accession numbers: KF214996–KF216452), data files are available from Dryad (http://dx.doi.org/10.5061/dryad.420h7), and trees are available on TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S14354).

Guide Trees, Delimitation, and Migration

The Cytb gene tree generated by BEAST recovered 11 well-supported milksnake lineages (Fig. 1a,c); 1 in the United States and Canada (referred to hereafter as Eastern), 2 in the United States (Western, *elapsoides*), 5 restricted to Mexico (Colima, Guerrero, Oaxaca, Puebla, and Sonora), 1 in Mexico and CA (Nicaragua), 1 exclusively in CA (Honduras), and 1 in CA and SA. These lineages are scattered throughout the tree and do not form a monophyletic group with respect to other *Lampropeltis* species (Fig. 1a). As in previous studies *L. alterna*, a species not traditionally allied with any of the delimited milksnake species based on morphology (Garstka 1982), was found to have mtDNA haplotypes similar (0.0–0.2% divergence between sequences) to those of milksnake populations from western Texas and northeastern Mexico (Western lineage). This resulted in all *L. alterna* samples being included within the Western lineage, along with milksnake populations from northeastern Mexico (Fig. 1a). One sample of *L. mexicana* from Nuevo León, Mexico (RB4) was also recovered as a member of the same Western milksnake lineage. Because it is the only individual of this species from this region (northern Sierra Madre Oriental) included in our study, we omitted it in further analyses. The coalescent-based analyses conducted here require multiple individuals ($\geq$2) for robust results (Heled and Drummond 2010; Zhang et al. 2011).

When BPP was run using the guide tree generated in *BEAST that consisted of the 11 Cytb milksnake lineages and *L. alterna*, all 11 lineages and *L. alterna* were recovered as distinct species with high support values (PP $\geq$ 95%) for the models with less conservative speciation priors (Table 3). However, the combination of a large ancestral population and shallow divergences resulted in lower support values for the divergences within some of the Mexican and Central American Cytb lineages (Table 3). Randomizations of individuals into the clades resulted in high support (PP $\geq$ 98%) of all nodes collapsed, indicating that BPP is identifying speciation events in the analyses.

Structurama indicated the highest PP (58%) for seven groups: six comprised of milksnakes, including *elapsoides*, Eastern/Western, Tamaulipas, Mexico (MX), CA, SA, and one distinct *L. alterna* group. The populations recovered by Structurama had individuals assigned to them with high support (PP $\geq$ 98%; see online Appendix 1), and resulted in a lower number of groups than in the Cytb tree. The genotypic cluster of *L. alterna* identified by Structurama was not nested within any groups of milksnake. Additionally, Structurama recovered a milksnake group that was part of the Western lineage in the Cytb tree. This group, the Tamaulipas milksnake lineage, was also found to be distinct in the BPP analyses (PP = 100%), despite not being a separate lineage in the Cytb gene tree. Results from all BPP analyses based on the Structurama groupings indicate high support for the six milksnake groups and *L. alterna* as separate species (Table 3).

Based on these results, we ran three additional BPP runs, using the same parameters previously specified, to explicitly test 1) whether *L. alterna* could be delimited from the Western milksnake lineage, 2) whether the Tamaulipas milksnake lineage could be delimited from the Western milksnake lineage, and 3) whether the Eastern and Western milksnake lineages could be delimited from one another. All of these runs resulted in PP = 100% for each as a separate species (Table 3).

Migrate-n analyses found little migration between the inferred milksnake lineages, indicating low levels of gene flow between the putative species, suggesting that BPP should give robust results with respect to delimitation (Zhang et al. 2011). Results were nearly identical between the independent runs and so results from one run are shown (Table 2). Although still relatively low (0.45 individuals/generation, HPD = 0.06–1.22), migration from the Eastern milksnake lineage into the Western milksnake lineage was

TABLE 2. Mean Number of Migrants per Generation between Geographically Adjacent Milksnake Lineages + *Lampropeltis alterna* Using Migrate v.3.2.16

From Lineage	To Lineage	Migrants Per Generation
Eastern	Western	0.45 (0.06–1.22)
Western	Eastern	0.27 (0.02–0.82)
Eastern	*L. elapsoides*	0.03 (0.00–0.17)
L. elapsoides	Eastern	0.13 (0.00–0.43)
Western	*L. elapsoides*	0.03 (0.00–0.17)
L. elapsoides	Western	0.22 (0.01–0.70)
Western	*L. alterna*	0.02 (0.00–0.14)
L. alterna	Western	0.17 (0.01–0.55)
Tamaulipas	*L. alterna*	0.03 (0.01–0.21)
L. alterna	Tamaulipas	0.11 (0.01–0.35)
Western	Tamaulipas	0.15 (0.00–0.53)
Tamaulipas	Western	0.30 (0.01–0.88)
Tamaulipas	MX	0.03 (0.00–0.15)
MX	Tamaulipas	0.04 (0.00–0.09)
MX	CA	0.06 (0.00–0.22)
CA	MX	0.06 (0.00–0.30)
CA	SA	0.03 (0.00–0.26)
SA	CA	0.04 (0.00–0.21)

Note: Results are based on 11 nuclear loci and a mean generation time for *Lampropeltis* of 2.5 years. The 95% highest posterior density is shown in parentheses.

highest, followed by migration from the Tamaulipas milksnake lineage into the Western milksnake lineage (0.31 individuals/generation, HPD = 0.01–0.88). The lowest migration rates were found among the MX, Tamaulipas, CA, and SA milksnake lineages (≤0.06 individuals/generation, HPDs = 0.00–0.30). There was also little migration between *L. alterna* and the Western milksnake lineage (≤0.17 individuals/generation, HPDs = 0.00–0.55) and *L. alterna* and the Tamaulipas milksnake lineage (≤0.11 individuals/generation, HPDs = 0.01–0.35).

The network constructed from the scnDNA data set resulted in similar groups as observed in the Structurama results, with high bootstrap support values (≥70) for the *L. alterna*, *L. t. elapsoides*, and Tamaulipas groups (Fig. 2). No statistical support for recombination was recovered (all loci $P \geq 0.06$). An individual from Guerrero, MX (AMNH 21940), and one from Veracruz, MX (AMNH 22617) that were classified in the CA group by Structurama were found among MX lineage individuals. The network showed a lack of differentiation with respect to the SA, MX, and CA clades, as the SA and CA clades were clustered within MX individuals (Fig. 2). The Eastern and Western clades formed two groups within the network, but with one Eastern individual (FTB 442 from New York) among the Western individuals (Fig. 2).

Species Trees

Stationarity for the species tree was determined by visually examining the traces from Tracer v.1.5 (Rambaut and Drummond 2007) and assuring that the effective sample sizes for crucial parameters were high (>200) and nearly identical topologies and dates were estimated between the four runs. This suggests stability with respect to the species relationships and timing of divergence events, and the first 25% of samples were discarded as burnin. As results were similar among runs, we show the resulting species tree chronogram from one *BEAST analysis (Figs. 1 and 3); this species tree was used for all subsequent comparative analyses. Results from the *BEAST analysis, using the delimited species (Table 4), indicate these milksnake species do not form a monophyletic species complex and the species tree topology is incongruent with both the Cytb gene tree (Fig. 1) and the scnDNA concatenated tree (Fig. 3).

We did not partition the milksnake lineages recovered exclusively from the Cytb gene tree in any species tree analyses. This was due to the lower support values under the more conservative BPP speciation model for the Cytb lineages (Table 3) and, as discussed in more detail later, the relatively small sample sizes for several of the clades (∼three individuals in some, see online Appendix 1). Unlike the Cytb tree, the scnDNA species-tree placed *L. alterna* in a more traditional arrangement (based on morphological data) as the sister-group to *L. mexicana* (Gehlbach and Baker 1962), not any milksnake lineage (Fig. 1). The species tree supports (≥95% PP) two main clades that diverged ∼8 Ma (HPD 5.3–11.9); one clade containing *L. calligaster*, *L. extenuata*, and the *L. getula* complex, the other containing all the milksnake lineages and a well-supported (≥95% PP) clade of mountain and Mexican kingsnakes (Figs. 1 and 3). Based on our analyses, speciation within *Lampropeltis* took place mostly during the Pleistocene (producing 15 extant species), with five species originating in the Pliocene and only one in the Late Miocene (Fig. 3).

Introgression

The results from JML and the coalescent simulations conducted in R both support mtDNA introgression between both the Western and Tamaulipas milksnake lineages with *L. alterna*. The JML analysis, based on 5000 simulations, detected introgressed Cytb sequences for *L. alterna* and Western lineage milksnakes ($P = 0.0002$) and *L. alterna* and Tamaulipas lineage milksnakes ($P = 0.009$); no other loci showed significant evidence of introgression. The gene trees from RAxML resulted in two reciprocally monophyletic nuclear gene trees (LATCL and VIM56) for *L. alterna* and Western lineage milksnakes and one reciprocally monophyletic nuclear gene tree (2CL8) for *L. alterna* and Tamaulipas lineage milksnakes. Results from the simulations using the method of Rabosky et al. (2009) indicated that the probability of obtaining two monophyletic nuclear loci and a nonmonophyletic mtDNA locus for *L. alterna* and Western lineage milksnakes due to incomplete lineage sorting was 0.002. Similarly, the probability of obtaining one monophyletic nuclear locus and a nonmonophyletic mtDNA locus for *L. alterna* and Tamaulipas lineage milksnakes due to incomplete lineage sorting was

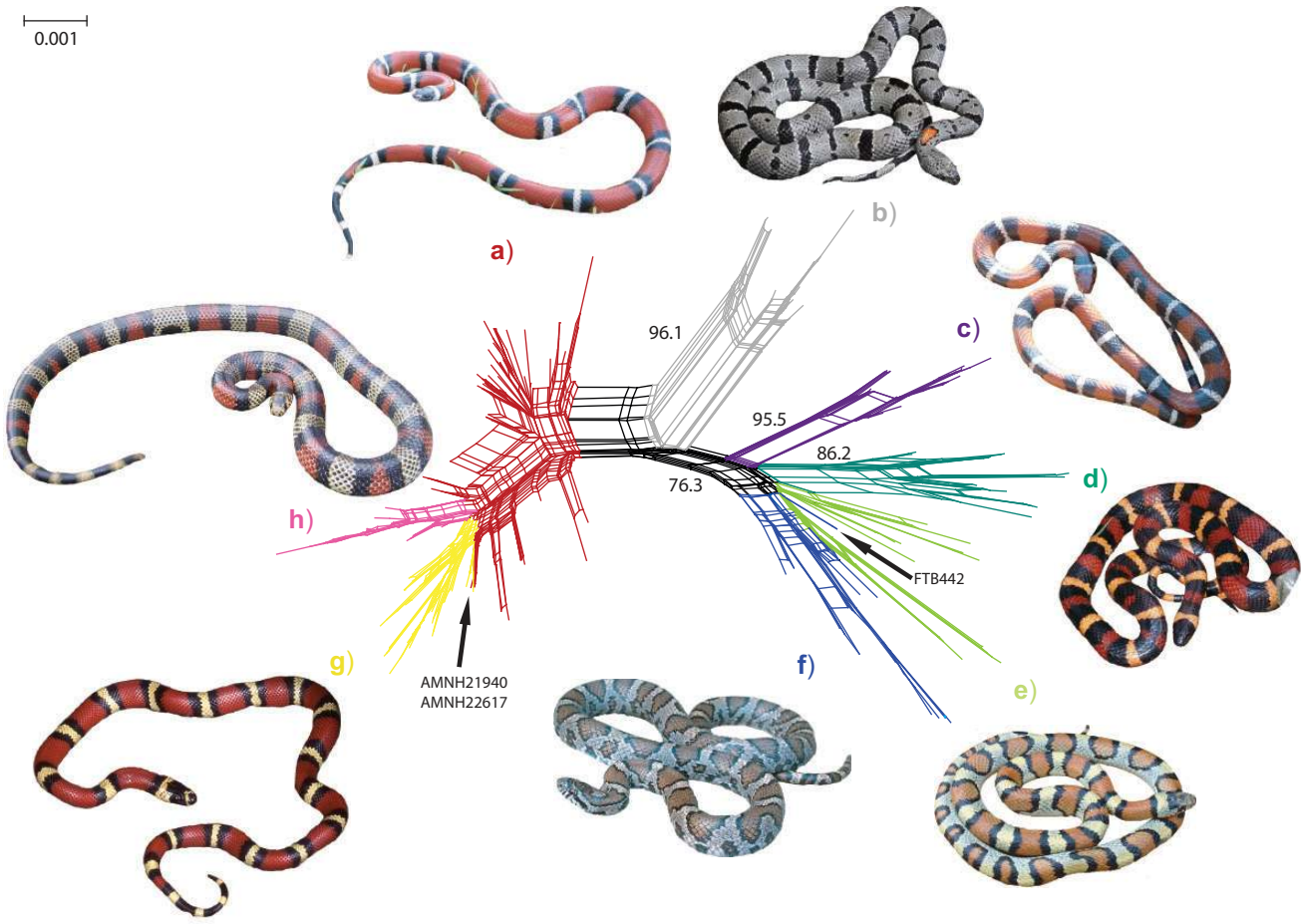

FIGURE 2. Nuclear gene network from Splitstree showing relationships for the delimited milksnake species (n = 82) and *Lampropeltis alterna* (n = 7). Species names correspond to those used in the species tree and are as follows: a) *L. polyzona*, b) *L. alterna*, c) *L. elapsoides*, d) *L. annulata*, e) *L. gentilis*, f) *L. triangulum*, g) *L. abnorma*, and h) *L. micropholis*. Bootstrap support values for major nodes ≥70 are indicated, as are three individuals that were placed within a lineage to which they do not belong.

0.012; thus these patterns are more likely due to introgression rather than deep coalescence in the mtDNA genome.

Concatenated scnDNA Tree and Timing of Diversification

Stationarity for the concatenated tree was again determined by visualizing inspecting for stationarity and assuring ESS > 200 for crucial parameters, and the first 25% of samples were discarded as burnin. Both the topology and divergence times differed between the concatenated and species trees. Comparing the species tree with the concatenated tree resulted in a Robinson-Foulds distance of 14, indicating that a total of 14 bipartitions are found in one tree but not in the other, although this score may be amplified by taxa whose placement is poorly supported in both trees (e.g., *L. t. elapsoides*).

Divergence times estimated using the scnDNA concatenated tree (Fig. 3) resulted in mean estimates that were significantly older when compared with the species tree (Wilcoxon signed-rank test, $Z = 3.883$, df = 20, $P < 0.001$), with the linear regression of branching-time error against species-tree branching time indicating significantly higher error at younger nodes ($t = -4.298$, df = 18, $P < 0.001$, $r = -0.5064$; Fig. 4). The divergence times from the concatenated tree (Fig. 3) showed a similar number of extant species diverging during the Pleistocene (nine species) and the Pliocene (10 species), and two species originating in the Miocene. The species tree resulted in a nonsignificant γ (–1.11; $P = 0.13$), indicating that diversification has been constant through time (Pybus and Harvey 2000). In contrast, the scnDNA concatenated tree resulted in a significantly negative γ (–2.44; $P < 0.01$), meaning that most divergence events took place further in the past, and diversification has slowed down toward the present.

The best-fitting diversification models for the species tree generally differed from those for the concatenated tree. The exception to this were the extinction models, where DD + E had the lowest AICc values for both trees, indicating that diversification is diversity dependent, although the estimated speciation rates, extinction rates, and carrying capacity differed between the two (Table 5). The best standard model for the species tree was Yule2, which indicated a decrease in speciation rates at ~1 Ma

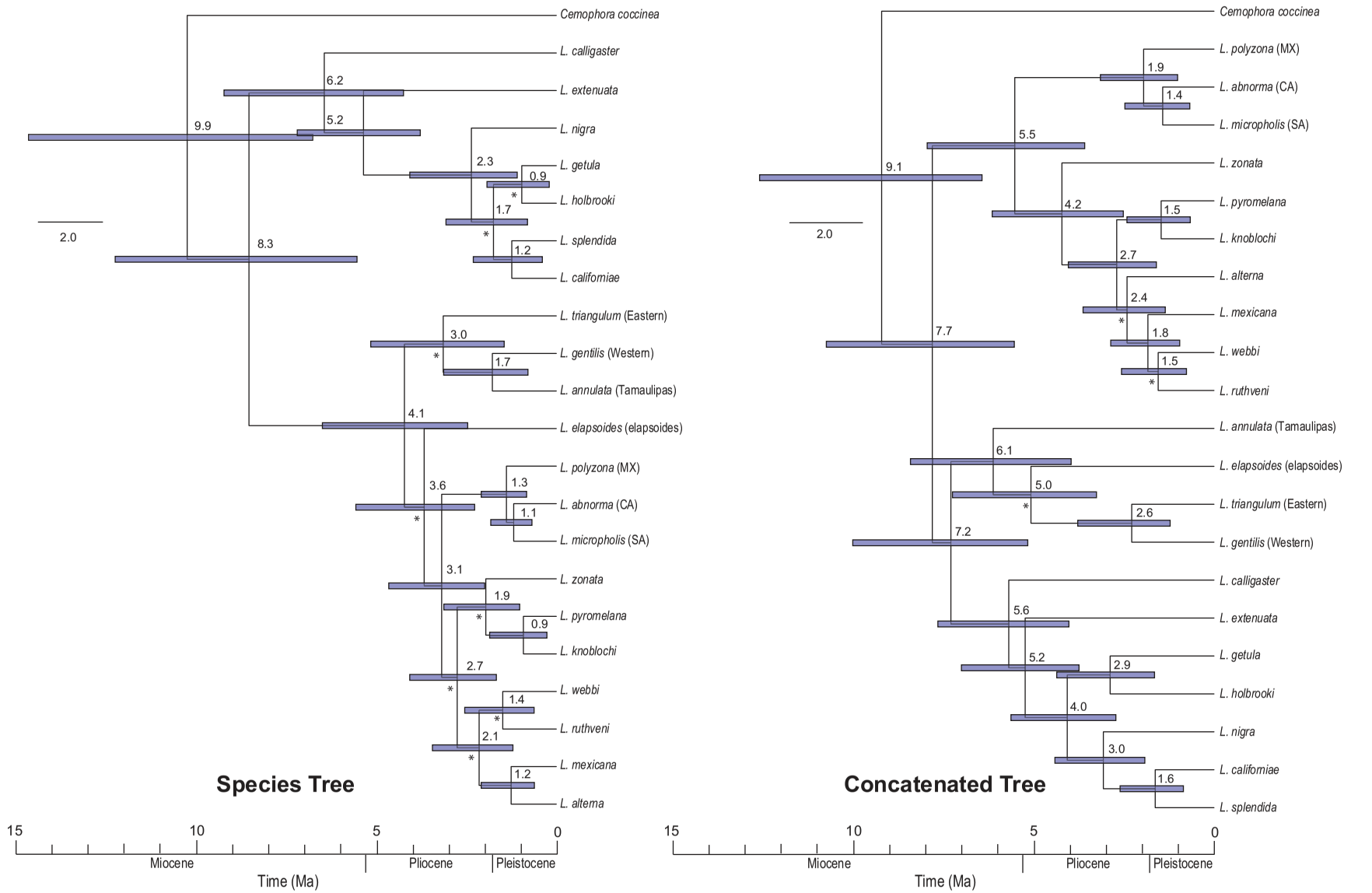

FIGURE 3. Original species-tree chronogram generated in *BEAST for *Lampropeltis* (left) and concatenated-scnDNA chronogram from BEAST showing mean divergence times (Ma) and including error bars indicating the 95% highest posterior density. Support values <95% are indicated by *.

TABLE 3. Posterior Probabilities from BPP Analyses Based on the Cytb Guide Tree, the Structurama-Based Guide Tree, and Additional BPP Runs to Verify Support for Several Taxa as Delimitable Species

	Cytb Guide Tree				Structurama Guide Tree				Additional Tests		
	BPP1 (%)	BPP2 (%)	BPP3 (%)		BPP1 (%)	BPP2 (%)	BPP3 (%)		BPP1 (%)	BPP2 (%)	BPP3 (%)
L. alterna	100	100	100	*L. alterna*	100	100	100	*L. alterna*	100	100	100
Colima	<90	≥95	100	CA	100	100	100	Eastern	100	100	100
Eastern	100	100	100	Eastern/Western	100	100	100	Tamaulipas	100	100	100
L. elapsoides	100	100	100	*L. elapsoides*	100	100	100	Western	100	100	100
Guerrero	≥95	≥95	100	MX	100	100	100	N/A	–	–	–
Honduras	<95	≥95	100	SA	100	100	100	N/A	–	–	–
Nicaragua	<95	≥95	100	Tamaulipas	100	100	100	N/A	–	–	–
Oaxaca	100	≥95	100	N/A	–	–	–	N/A	–	–	–
Puebla	<95	100	100	N/A	–	–	–	N/A	–	–	–
SA	100	100	100	N/A	–	–	–	N/A	–	–	–
Sonora	<90	≥95	100	N/A	–	–	–	N/A	–	–	–
Western	100	100	100	N/A	–	–	–	N/A	–	–	–

Note: Results are based on three runs from each parameterization. The parameters for ancestral population size and root age are as follow: BPP1 = large populations and deep divergences, BPP2 = small ancestral populations and shallow divergences, and BPP3 = large ancestral populations, and shallow divergences.

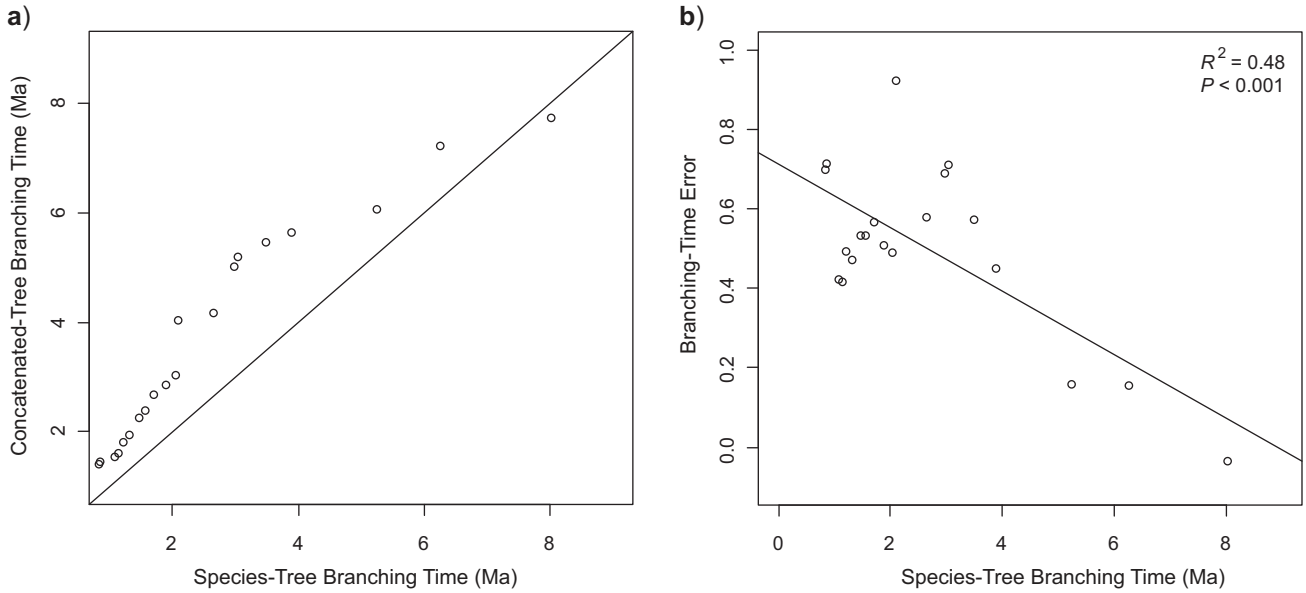

FIGURE 4. Plots showing a) the species tree branching times versus the scnDNA concatenated tree branching times for *Lampropeltis*, with a line through the origin illustrating that the mean times of the concatenated tree are generally older, and b) a linear regression of the scaled-branching time error against the species-tree branching times, including the R^2 and P values.

from $\lambda = 0.440$ to $\lambda = 0.090$ and the best coalescent model was Model 6, indicating expanding diversity through time with no extinction and exponentially declining speciation (Table 5). For the concatenated tree, the best-fitting standard model was DDL, which indicates that diversification is density dependent, and the best-fitting coalescent model was Model 5, which is equivalent to a Yule process with a constant birth-rate through time (Table 5).

DISCUSSION

We find that species diversity has been greatly underestimated for *Lampropeltis* and that using a species-tree approach to estimate divergence times results in more recent speciation times when compared with gene-tree methods or studies with incomplete taxon sampling (Pyron and Burbrink 2009a, 2009d; Burbrink and Pyron 2010). Our results suggest that diversification peaked during the Pleistocene and Pliocene for the genus *Lampropeltis* (Fig. 3) and we detect no slowdown in diversification for this genus using the γ statistic, although the best-fitting diversification models do indicate that speciation rates have declined over time (Table 5). Our results underscore the necessity to include all available extant taxa in species-tree analyses and the importance of using multispecies coalescent-based methods to infer phylogenies for comparative analyses.

TABLE 4. Lineages of Milksnake and Their Corresponding Species Designation and General Geographic Area

Lineage	Species name	Main geographic extent
Eastern	*L. triangulum* (Lacépède 1788)	Eastern USA, Southeastern Canada
Western	*L. gentilis* (Baird and Girard 1853)	Western USA
L. elapsoides	*L. elapsoides* (Holbrook 1838)	Southeastern USA
Tamaulipas	*L. annulata* Kennicott 1861)	Northeastern Mexico
MX	*L. polyzona* Cope 1861	Western and Central Mexico
CA	*L. abnorma* (Bocourt 1886)	South-Central Mexico to Eastern Costa Rica
SA	*L. micropholis* Cope 1861	Western Costa Rica south to Ecuador

Note: More detailed ranges are shown in Figure 1. Species names are taken from the oldest known subspecies name within the range of that lineage as reported by Williams (1988).

Species Delimitation

Our analyses support the existence of seven distinct species previously considered to be *L. triangulum*, which we propose should be formally recognized. These seven taxa were all originally described as full species based on morphology (e.g., size, body form, color/pattern) before being synonymized with *L. triangulum* and are as follows: *L. triangulum* (Lacépède 1788), *L. gentilis* (Baird and Girard 1853), *L. elapsoides* (Holbrook 1838), *L. annulata* Kennicott 1861, *L. polyzona* Cope 1861, *L. abnorma* (Bocourt 1886), and *L. micropholis* Cope 1861. Species designations and distributions are in Table 4 and Figure 1 and detailed in Appendix 2. We refer to the seven milksnake species by the names presented earlier throughout the remainder of the discussion. Future studies that include a morphological assessment of specimens and updated diagnoses of these species would be a valuable complement to the species delimitation presented here.

The mtDNA gene tree and results from the Structurama analysis both reveal previously unrecognized diversity within *L. triangulum* (*sensu lato*). The mtDNA tree indicates 11 lineages, whereas Structurama finds 6 distinct milksnake groups. However, the relatively small number of individuals in some of the Cytb clades (online Appendix 1) makes the designation of all 11 Cytb lineages as distinct species premature. Simulations using BPP show that the number of samples from a population affects the accuracy of the program and that analyses with lower numbers of individuals give less robust results (Zhang et al. 2011). In addition, the support for these lineages when using the large ancestral population size and shallow divergence speciation model was generally low (<95%). Together these results suggest that more sampling is needed across Middle America to better estimate diversity and clarify relationships.

Although we acknowledge that there may be additional species of milksnake, particularly in Middle America, we conservatively recommend that seven milksnake lineages be recognized as full species (Table 4). These species represent the six milksnake groups found by Structurama that were also supported in the BPP analyses (Table 3), which includes the Eastern and Western lineages found in the Cytb tree, delimited with high support in BPP (100%; Table 3). The nuclear network (Fig. 2) also indicates that these seven milksnake groups are distinct from *L. alterna*, although there is some mixing of individuals from the CA and MX lineages (AMNH 21940 and AMNH 22617) and the Eastern and Western lineages (FTB 442), potentially due to incomplete lineage sorting within the nuclear loci or low levels of migration that were not detectable using Migrate-n analyses (Table 2).

Phylogeny

The seven species of milksnakes do not form a single monophyletic group in the species-tree analysis (Fig. 1b), supporting previous molecular work (Bryson et al. 2007; Harper and Pfennig 2008; Pyron and Burbrink 2009a, 2009d). However, these previous studies used concatenation methods and mtDNA to infer phylogeny (Bryson et al. 2007; Harper and Pfennig 2008; Pyron and Burbrink 2009a, 2009d), and as a result, all previous phylogenies are at odds with our species tree. Interestingly, Blanchard's (1921) revision of *Lampropeltis* based on morphology suggests relationships largely congruent with our study, such as a close relationships among tri-colored *Lampropeltis* species (e.g., milksnakes: *L. elapsoides*, *L. gentilis*, *L. micropholis*, *L. polyzona*, *L. triangulum*; mountain kingsnakes: *L. pyromelana*, *L. zonata*; and Mexican kingsnakes: *L. mexicana*, *L. ruthveni*) and between the *L. getula* group and *L. calligaster*.

In addition to a lack of support for a monotypic milksnake, none of the commonly recognized milksnake subspecies included here, with the exception of the scarlet kingsnake (*L. elapsoides*), are reciprocally monophyletic taxa. This finding is further evidence that using highly variable color patterns in snakes may be unreliable as a character for defining and naming taxa (Burbrink et al. 2000; Cox et al. 2012). There is discordance between the species tree and concatenated scnDNA gene tree (RF=14), despite using the same loci and terminal taxa. This further underscores that, even when the same species are delimited and included in analyses, gene trees are not equal to multispecies coalescent-based species trees with respect to temporal or topological congruence.

Mitochondrial Introgression

Our analyses strongly support mtDNA introgression between species of *Lampropeltis* as one reason for discordance between mtDNA and scnDNA analyses. Although mtDNA is frequently used to infer phylogeographic patterns, our study adds to

the growing evidence that for many major groups of vertebrates, mtDNA can mislead phylogenetic inference (Brumfield et al. 2003; Bossu and Near 2009; Spinks and Shaffer 2009; Bryson et al. 2010; Leaché 2010; Pasachnik et al. 2010; Waters et al. 2010; Fontenot et al. 2011; Roos et al. 2011; Yu et al. 2011; Lee et al. 2012; Veith et al. 2012). Relationships within *Lampropeltis* inferred using mitochondrial genes (Cytb, Fig. 1a; ND4, Bryson et al. 2007) result in well-supported gene trees that conflict with the species tree (Fig. 1b). The kingsnake *L. alterna* is typically allied with *L. mexicana* based on morphology (Gehlbach and Baker 1962), yet all individuals sampled have mtDNA haplotypes similar or identical to geographically proximate milksnakes. A similar pattern seemingly exists for *L. mexicana* from the northern Sierra Madre Oriental (Bryson et al. 2007).

Two possible reasons for the similar mtDNA haplotypes found in our study among *L. gentilis*, *L. alterna*, *L. annulata*, and the single *L. mexicana* specimen are incomplete lineage sorting or introgression among species. Incomplete lineage sorting is most likely to occur when internodes are short, due to rapid speciation events (Moore 1995). This does not appear to be the case for *L. alterna* and the two sister taxa *L. gentilis* and *L. annulata*, which share a most recent common ancestor ~5 Ma in the species tree. Our JML analyses that specifically differentiate between incomplete lineage sorting and genetic introgression demonstrate that introgression best explains the mtDNA pattern with respect to *L. alterna* and *L. gentilis* and *L. annulata*. However, the JML analysis did not find any evidence of introgression between *L. gentilis* and *L. annulata.* It is possible, given their sister relationship, and hence recent divergence on the species tree, that the failure to sort has resulted in the similarity between mtDNA haplotypes of *L. gentilis* and *L. annulata*. The gray-banded kingsnake *L. alterna* was considered a subspecies of *L. mexicana* for decades (Gehlbach and Baker 1962; Garstka 1982) and has been assumed to be closely related to *L. mexicana* since the 1940s (Smith 1942, 1944; Gehlbach and Baker 1962; Garstka 1982). Gene flow or hybridization between *L. alterna* and *L. mexicana* from the nearby northern Sierra Madre Oriental may be responsible for the seemingly introgressed milksnake mtDNA in our sample of *L. mexicana* from this region. More samples of *L. mexicana* are necessary to help distinguish between this and competing explanations for this pattern.

Although mtDNA typically sorts faster than most nuclear genes, making it a seemingly good choice for species delimitation, it may be more vulnerable to introgression for multiple reasons (reviewed by Funk and Omland 2003) and similar to our findings, mitochondrial introgression has been frequently reported in other taxa (Bossu and Near 2009; Bryson et al. 2010; Nevado et al. 2011). A recent review (Petit and Excoffier 2009) also finds that for species with higher levels of male versus female dispersal, markers inherited maternally (e.g., mtDNA) have higher rates of introgression compared with those inherited through both sexes. Male-biased dispersal appears to be common in snakes (Rivera et al. 2006; Keogh et al. 2007; Dubey et al. 2008; Welsh et al. 2010; Pernetta et al. 2011), and this may partially explain the introgression between the species examined here. Although our analyses of introgression suggest that there has been mtDNA introgression between *L. gentilis* and *L. annulata* with *L. alterna*, the Migrate-n analyses show little evidence for ongoing migration (based on the scnDNA) among these taxa (Table 2), suggesting that the mtDNA introgression is likely historical rather than contemporary.

Timing and Processes of Diversification

Our time-calibrated species tree indicates that *Lampropeltis* originated in the Miocene, similar to previous studies (Pyron and Burbrink 2009d). However, unlike Pyron and Burbrink (2009d), our expanded species-tree shows the majority of divergences for extant *Lampropeltis* species occurred during the Pleistocene and Pliocene (Fig. 3). This is likely for two reasons; first, our tree has more than twice as many terminal taxa for the genus, with 21 species of *Lampropeltis* compared with 10 in the previous study. The inclusion of these additional species results in 11 new divergence events occurring mostly in the Pleistocene (73% of new nodes). These results are similar to studies of other vertebrates revealing high amounts of Pleistocene diversification (Rand 1948; Avise 2000; Hewitt 2000, 2004; Johnson and Cicero 2004; Rull 2006; Beheregaray 2008; Koscinski et al. 2008; Zarza et al. 2008; Daza et al. 2009), suggesting a role for glacial-interglacial cycles in the diversification of *Lampropeltis*. We recognize there may still be cryptic diversity to be discovered within *Lampropeltis* (e.g., *L. calligaster* , *L. mexicana*, and *L. zonata*; see Myers et al. 2013), but additional taxa would likely result in the accumulation of more shallow divergence times, because cryptic taxa would diverge within the already recognized species.

Second, our use of a species tree and not a gene tree should result in younger divergence times (Edwards and Beerli 2000; Carstens and Knowles 2007; McCormack et al. 2010; Burbrink and Pyron 2011). Our analyses support this, with the scnDNA concatenated tree showing most diversification during the Pliocene (55% of nodes) and Miocene (80% of nodes) and the species tree indicating the majority of speciation events occur more recently during the Pleistocene (45% of nodes) and the Pliocene (45% of nodes). There is a significant difference in the mean divergence times between these trees, which increases as nodes become younger (Figs. 3 and 4). Unlike the species tree, the older divergences from the scnDNA concatenated tree also contribute to a γ value suggesting early diversification for *Lampropeltis*, further illustrating the problems that can result from relying on gene trees in diversification analyses (Burbrink and Pyron 2011).

Although the γ statistic indicated node density to be constant through time for the species tree, the best-fitting diversification models for the species tree found the

TABLE 5. Best Diversification Model and Its Corresponding AICc Value for Both the scnDNA Concatenated Tree and the Species Tree for Full-Likelihood Standard Models, Approximate-Likelihood Coalescent Models, and Models That Include Extinction

Models	Concat. tree	AICc	λ	μ	*K*	Species tree	AICc	λ	μ	*K*	sp	r1	r2
Standard model	DDL	4.815	0.68	NA	22	Yule2	−1.555	NA	NA	NA	1.080	0.440	0.090
Coalescent model	Model 6	−6.542	NA	NA	NA	Model 5	−16.590	NA	NA	NA	NA	NA	NA
Extinction model	DD + E	7.56	0.68	0.000003	22	DD + E	2.888*	0.57	0.05	28	NA	NA	NA

Note: The applicable parameter estimates for each model included are as follows: λ, speciation rate; μ, extinction rate; *K*, carrying capacity; r1, speciation rate 1; r2, speciation rate 2; sp, shift point; NA, non-applicable parameters are indicated for the models.
*For the species tree, the next best AICc value was 3.099 for BD, <1 from the DD + E model.

speciation rates of *Lampropeltis* have decreased through time (Yule2, coalescent model 6, DD + E; Table 5). This mirrors results reported by Burbrink and Pyron 2010, who found that density-dependent and Yule2 models explained diversification of Lampropeltini equally well. However, the concatenated tree gives conflicting results, with both constant speciation rate (coalescent model 6) and variable-rate density-dependent models being supported (DDL, DD+E; Table 5). It is possible that the low number of taxa in *Lampropeltis* does not permit us to differentiate confidently among various diversification models (O'Meara et al. 2006; Rabosky 2006; Boettiger et al. 2012). Our analyses of *Lampropeltis* further illustrate that even the terminal taxa are identical, concatenated gene-trees will not necessarily infer the same models of diversification as the species tree, at least with a small number of taxa.

CONCLUSIONS

Our results suggest that species delimitation prior to species-tree inference or analyses that use phylogenies will provide more robust results with respect to topology, timing, and tempo of diversification compared with studies that are missing taxa and/or rely on gene-tree approaches. By delimiting previously unrecognized milksnake species within *Lampropeltis* and including other recently recognized species within the genus, we provide both a well-supported species tree and diversification time estimates for *Lampropeltis*. We find that speciation of *Lampropeltis* was highest during the Pleistocene and Pliocene, indicating that recent glacial cycles may have been important for diversification in this group. The differences between the mtDNA gene trees, species delimitation results, and species tree analyses, particularly with respect to *L. alterna*, further underscore the problems with relying on phylogenetic patterns from gene trees, which may result in misleading species recognition and taxonomic composition.

Our recommendation for the elevation of seven milksnake species provides a better estimate of the diversity not only within Lampropeltini but also for New World squamate diversity. More generally, we show that the omission of unrecognized species diversity and the use of concatenated gene trees have serious and far-reaching repercussions for the correct inference of phylogenetic relationships and the dynamics of recent speciation events. Even for groups that are fully sampled with regard to currently described species, the common inference of early bursts of speciation may be driven in many cases by inadequate taxon sampling at the phylogeographic level (where hidden species diversity is most likely to be found), and the use of gene-tree based divergence-time estimates. Missing taxa not only affect the tree topology but also contribute to misleading inferences with respect to divergence time estimation and the downstream analyses that rely the accurate timing of diversification. This intersection between phylogeography, systematics, and species diversification analyses has not often been examined, yet is demonstrated here to have a crucial impact on the primary goals of systematics and evolutionary biology, including species delimitation, species-tree estimation, and the inference of evolutionary processes of speciation using phylogenetic comparative methods.

SUPPLEMENTARY MATERIAL

Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.420h7.

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APPENDIX 1

Twelve loci used for *Lampropeltis* and PCR/sequencing protocols. The overall PCR conditions for all loci were the same with the exception of the annealing temperature (discussed in the Appendix Table) and used GoTaq Green MasterMix (Promega Corp.) according to the manufacturer's specifications, with a 90-s extension time. The PCR products were cleaned using 1 μL of ExoSap-IT (USB Corp.) per 10 μL of PCR product. The sequencing reaction consisted of 2 μL Beckman-Coulter DTCS, 1 μL primer (10 μM), 2 μL template, and 5 μL deionized water. All loci are nuclear with the exception of cytochrome *b* (mitochondrial); GAD2 intron 15 is from the Z-chromosome. If the primer was developed for this project, the sequence is included. Internal primers used

Locus	Protein coding	Length (bp)	Primer name/sequence	Primer source	Annealing temperature (°C)
Cytochrome *b*	Yes	1,117	H14910	Burbrink et al. 2000	49
			THRSN2	Burbrink et al. 2000	49
			MxTriangF: 5′-CGA TTC TTT GCC YTA CAC TT-3′*	Developed for this project	48
			MxTriangR: 5′-GAC TGA TAT GGR TGG AAT GGA-3′*	Developed for this project	48
			Triangulum1F: 5′-ACA GAA YTA ACY AAC TGA CT-3′*	Developed for this project	43.9
			Triangulum2R: 5′-ATT TTR TCR ATA TCH GAG TTT GT-3′*	Developed for this project	43.3
NT3	Yes	481	NT3-F3	Noonan and Chippindale 2006	51
			NT3-R4	Noonan and Chippindale 2006	51
PRLR	Yes	552	PRLR-F1	Townsend et al. 2009	48
			PRLR-R3	Townsend et al. 2009	48
GAD2 intron 15	No	541	EST GAD2 15F: 5′-CAC ACA AAT GTY TGC TTC TGG-3′	Developed for this project	48.3
			EST GAD2 16R: 5′-ATG CGG AAR AAA TTG ACC TTG TC-3′	Developed for this project	48.3
			GAD15_16 intF: 5′-ACC TCA CAA TGA AGA TTT GTG-3′*	Developed for this project	46
			GAD15_16 intR: 5′-GTG TAG ATG CTA CTG AAG CAA AGT C-3′*	Developed for this project	48.6
NAV intron 5	No	561	NAV5F	Geffeney et al. 2005	55
			NAV6R	Geffeney et al. 2005	55
SPTBN intron 1	No	839	SPTBN1F APR-2010: 5′-TTG GTC GAT GCC AGT TGT A-3′	Developed for this project	48.5
			SPTBN1R APR-2010: 5′-CAG GGT TTG TAA CCT KTC CA-3′	Developed for this project	48.5
			SPTBN1 interF: 5′-TTT CCT TTC CAT TCC TTC TTT C-3′*	Developed for this project	46
			SPTBN1 interR: 5′-GGC TGT CTG TTT GCA TCT TG-3′*	Developed for this project	49
Vimentin intron 5	No	584	Vim Exon 5F	Pyron and Burbrink 2009d	55
			Vim Exon 6R	Pyron and Burbrink 2009d	55
CL4	No	373	CL4 F: 5′-CGC CTA AAA CTA ACA GTA GG-3′	Developed for this project	45.5
			CL4 R: 5′-GTT CAG AGA GAT CTG ATT GC-3′	Developed for this project	45.5
LAT Clone	No	705	CL LAT F: 5′-CCA GTG TGC TGG AAT TCA G-3′	Developed for this project	45.5
			CL LAT R: 5′-TAT CTG CAG CAT TCA GGA-3′	Developed for this project	45.5
2CL3	No	429	2CL3 F: 5′-TGC TGA ACT AGC AGT CAT-3′	Developed for this project	45.5
			2CL3 R: 5′-GCT TTC CCA AGA GGA ATG AAA T-3′	Developed for this project	45.5
2CL4	No	376	2CL4 F: 5′-ACT GGC AGG ATC CAG AA-3′	Developed for this project	47
			2CL4 R: 5′-AAT CCA GCA GCC TTT GAC-3′	Developed for this project	47
2CL8	No	466	2CL8F: 5′-CCC TCA ATC TAG CCC AGT-3′	Developed for this project	48
			2CL8R: 5′-GAT TAG CAG GAA ACT CT-3′	Developed for this project	48

specifically for sequencing reactions are indicated with *. For samples that were old/degraded, the internal primer was combined with a flanking primer to sequence the locus in two parts, using the internal primer's annealing temperature for the PCR.

Appendix 2

Taxonomic revision of *Lampropeltis triangulum.* We conservatively interpret our results based on coalescent species-delimitation models using 5908 bp of multilocus data obtained from 276 milksnakes to indicate the presence of seven species of milksnake that were formerly classified as subspecies of *L. triangulum*. Later, we list these seven species elevated from subspecific status, and provide details regarding their taxonomy and an estimation of their distributional ranges based on our sampling. A map showing proposed ranges is also included in Figure 1 of the main manuscript. Although a complete morphological description is beyond the scope of this article, we include a basic description of each species with respect to color pattern synthesized from the most complete morphological treatise of milksnakes (Williams 1988). However, as mentioned in our main manuscript, we stress that using highly variable color and pattern in snakes is largely unreliable for defining and naming species. Milksnakes are considered a classic example of Batesian mimicry, and if true, a strong selection on color and pattern may be driving perceived differences between species as well as convergence. This has been aptly demonstrated with New World coralsnakes (Castoe et al. 2007), one of several probable Batesian models for milksnakes. Finally, we treat our recommended species designations as a working hypothesis. Certainly, our data unequivocally demonstrate that the continued recognition of 25 subspecies of *L. triangulum* improperly characterizes species diversity within this wide-ranging snake. The seven species we recognize capture most of the observed genetic diversity. Additional species of milksnake, particularly in Middle America, are possible and require further study. Distributional boundaries of the seven species are also not absolute and will require future modification.

Lampropeltis triangulum (Lacépède 1788)

The oldest subspecies within the proposed range of the Eastern lineage is the nominate subspecies, *L. t. triangulum*, with the holotype unknown from "America" but restricted to the vicinity of New York City (Schmidt 1953); in absence of a type specimen, an adult male from Westchester County, New York (American Museum of Natural History 31848) was designated previously for descriptive purposes (Williams 1988). We designate our Eastern lineage as *L. triangulum*. Subspecies synonymized with *L. triangulum* include *L. t. syspila* and *L. t. amaura* (in part).

Range: The range of this species includes the entire distribution for *L. t. triangulum* as described by Williams (1988) from Ontario, Canada, along the Georgian Bay, throughout southern Quebec, and east of Lake Huron, extending throughout southern Maine, south through New England and New York to North Carolina and the extreme northern Alabama and Georgia and west to eastern Minnesota. Subspecies synonymized under *L. triangulum* would include *L. t. syspila* and any suspected "intergrades" that occur in Alabama, Indiana, Iowa, Illinois, Kentucky, Missouri, Mississippi, Tennessee, and possibly Arkansas north of the Arkansas River, and some milksnakes that have fallen under the subspecies *L. t. amaura* in northeastern Louisiana (specifically in La Salle Parish).

Diagnosis: Based on the descriptions of *L. t. triangulum* and *L. t. syspila* (Williams 1988), *L. triangulum* has brown, grey, or red blotches bordered in black on a lighter colored background (grey or cream); these blotches do not extend onto the venter. The head pattern consists of a dark-colored V or Y that connects to the first body blotch or alternately, may have the anterior of the head partially or nearly covered in black pigment and the posterior of the head red.

Lampropeltis gentilis (Baird and Girard 1853)

The oldest subspecies within the proposed range of the Western lineage is *L. t. gentilis*, originally described as a distinct species, with the lectotype an adult male from Wheeler County, TX (United States National Museum 1853; Blanchard 1921). We designate that the Western lineage be recognized *L. gentilis*. Subspecies synonymized with *L. gentilis* include *L. t. celaenops*, *L. t. multistriata*, *L. t. taylori*, *L. t. amaura* (in part), and *L. t. annulata* (in part).

Range: The range of *L. gentilis* includes the entirety of the subspecies *L. t. gentilis*, as described by Williams (1988), and is found in the Panhandle of northern Texas, western Oklahoma, central and western Kansas, eastern Colorado, and south-central and southwestern Nebraska. The range of *L. gentilis* also includes the ranges of the following subspecies, which are synonymized with *L. gentilis*: *L. t. amaura* (part), found in western Texas, southeastern Oklahoma, Louisiana west of the Mississippi River, and southern Arkansas; *L. t. celaenops* in southeastern Arizona, New Mexico, and adjacent western Texas; *L. t. multistriata*, found in northwestern Nebraska, the western half North Dakota, northern Wyoming, and southern Montana; and *L. t. taylori* found in Utah, northern Arizona, and western Colorado. In addition, *L. gentilis* includes *L. t. annulata* from at least central Texas and *L. t. syspila* from Nebraska, Kansas, and Oklahoma.

Diagnosis: Based on the descriptions of the subspecies, it encompasses, following Williams 1988, *L. gentilis* has red- or orange-colored rings bordered by black on a light-colored background (white, cream, and yellow), with either the red/orange or black extending onto the venter.

The head is generally black and may have white mottling on the snout.

Lampropeltis elapsoides (Holbrook 1838)

***Lampropeltis elapsoides* was originally described as a species, with the holotype unknown, from South Carolina and Georgia (Williams 1988); in absence of a type specimen, an adult female from Alachua County, Florida (University of Florida 20546) was previously used for descriptive purposes (Williams 1988).** We continue to recognize *L. elapsoides* as a distinct species, following recent authors (Pyron and Burbrink 2009a, 2009b).

Range: The range of *L. elapsoides* remains the same as that of the subspecies, being found across the southeastern United States as far north as Virginia and Kentucky east of the Mississippi River and in eastern Louisiana. Suspected "intergrades" with *L. triangulum* from eastern Virginia to southern New Jersey are likely *L. triangulum* and not hybrids based on our migration analyses.

Diagnosis: Based on the description of *L. t. elapsoides* from Williams (1988), *L. elapsoides* has a body pattern of black, red, and yellow rings that extend completely across the venter. The head is red with black across the posterior of the parietal scales. The iris of the eye is red.

Lampropeltis annulata Kennicott 1861

The oldest known subspecies within the proposed range of the Tamaulipas lineage is *L. t. annulata*. *Lampropeltis annulata* was originally described as a distinct species, with the holotype from Matamoros, Tamaulipas, Mexico (Academy of Natural Sciences of Philadelphia 3613). We designate the Tamaulipas lineage as *L. annulata*. Subspecies synonymized with *L. annulata* include *L. t. dixoni*.

Range: Based on our sampling, *L. annulata* is found in the Mexican states of Nuevo León, Querétaro, and Tamaulipas. It is likely that this species is also found in Coahuila, eastern San Luis Potosi, and Hidalgo.

Diagnosis: Following the descriptions *L. t. annulata* and *L. t. dixoni* from Williams (1988), the body pattern of *L. annulata* consists of incomplete red rings that are interrupted by black rings that cross the venter. The head is black.

Lampropeltis polyzona Cope 1861

The oldest known subspecies within the proposed range of the Mexico lineage is *L. t. polyzona*, originally described as a species with the holotype an adult male from Jalapa, Veracruz, Mexico (Academy of Natural Sciences of Philadelphia 9770). We designate the Mexico lineage as *L. polyzona*. Subspecies synonymized with *L. polyzona* include *L. t. arcifera*, *L. t. conanti* (in part), *L. t. campbelli*, *L. t. nelsoni*, *L. t. polyzona* (in part), *L. t. sinaloae*, and *L. t. smithi*.

Range: Based on our sampling, *L. polyzona* is found in the Mexican states of Colima, Guerrero, Hidalgo, Jalisco, Puebla, Michoacán, Oaxaca, Sinaloa, Sonora, and Veracruz. It is likely that this species is also found in Guanajuato, Morelos, and Nayarit, and western San Luis Potosí.

Diagnosis: Based on the descriptions of the subspecies it synonymizes, from Williams (1988), *L. polyzona* has red- and light-colored body rings are complete or may interrupt by black pigment across the venter. There may be black ticking on the red- and light-colored scales. The snout may be mottled with white or have a light-colored band crossing the prefrontal–internasal border, with the remainder of the head black.

Lampropeltis abnorma (Bocourt 1886)

The oldest known subspecies within the proposed range of the CA lineage is *L. t. abnorma*, originally described as a species with the holotype an adult female from Alta Verapaz, Guatemala (Museum National d'Historie Naturelle 88-129). We designate the CA lineage as *L. abnorma*. Subspecies synonymized with *L. abnorma* include *L. t. blanchardi*, *L. t. conanti* (in part), *L. t. hondurensis*, *L. t. oligozona*, *L. t. polyzona* (in part), and *L. t. stuarti*.

Range: Based on our sampling, *L. abnorma* is found in southern Veracruz and southeastern Guerrero ranging south through Nicaragua, Honduras, and western Costa Rica. This species is possibly in southern Oaxaca, and likely Campeche, Chiapas, Quintana Roo, Tabasco, and Yucatan as well as Belize and El Salvador.

Diagnosis: Based on the descriptions of the subspecies, it comprises from Williams (1988), the body rings of *L. abnorma* are complete or the red- and light-colored rings may be interrupted by black pigment. The red- and light-colored scales may be tipped with black. The head is black or may have a light-colored band crossing the internasals and prefrontals.

Lampropeltis micropholis Cope 1861

The oldest known subspecies within the proposed range of the SA lineage is *L. t. micropholis*, originally described as a species with the holotype from Panama (Academy of Natural Sciences 3427). We designate the SA lineage as *L. micropholis*. Subspecies synonymized with *L. micropholis* include *L. t. gaigeae* and *L. t. andesiana*.

Range: Based on our sampling, *L. micropholis* ranges from eastern Costa Rica, throughout Panama, and south to Ecuador. It is likely found in Colombia and possibly Venezuela.

Diagnosis: Following the descriptions of the subspecies it synonymizes, from Williams (1988), adult *L. micropholis* from Costa Rica and western Panama may have all pattern obscured by black pigment, although in younger/smaller individuals, there is a white band across the prefrontal. In specimens from other locales, the body rings extend across the venter. The snout is

white with black posterior margins. Red and white scales have extensive black ticking.

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