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Short Communication

Neogene diversification and taxonomic stability in the snake tribe Lampropeltini (Serpentes: Colubridae)

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1. Introduction

The impact of climate change during the Pleistocene on organisms living in temperate areas of the world has been documented for more than fifty years (Rand, 1948). A major impact of Pleistocene glacial cycles on vertebrates in temperate zones is hypothesized to be the isolation of species into allopatric populations (Avise and Walker, 1998). Refugial isolation and subsequent lineage formation during the Pleistocene has been invoked as a primary mechanism resulting in modern species diversity (Johnson and Cicero, 2004). Under the Pleistocene speciation model, these glacial cycles acted as a 'species pump,' producing the majority of extant organisms inhabiting temperate areas. These arguments, usually focused on dating the origin of sister species using a fixed rate of divergence in birds, have suggested that the majority of speciation events occurred in the Pleistocene (Johnson and Cicero, 2004; Weir and Schluter, 2007). In a more limited study, Avise et al. (1998) suggested that the Pleistocene was important for speciation in reptiles, amphibians, and fish as well.

In contrast to the Pleistocene diversification model, it has been shown that many speciation events in birds occurred much earlier than the Pleistocene (Zink and Slowinski, 1995; Klicka and Zink, 1997), potentially calling into question the effects of glacial cycles on the formation of species. Unfortunately, most of these studies have invoked a molecular clock using a fixed rate of mtDNA substitution over time, and thus did not consider the impact of molecular rate variation for assessing error in divergence dates. The effect of Pleistocene climatic cycles on speciation has not been investigated in other large groups of vertebrates, particularly those that may be more sensitive to the effects of glacial cycles, such as ectotherms like reptiles. We investigate these patterns in a major New World (NW) group of colubroid snakes, the tribe Lampropeltini, inferring a robust phylogeny for the group and estimating the timing of speciation and diversification. We use multiple nuclear and mitochondrial genes and 'relaxed phylogenetics' methods (Drummond et al., 2006) to estimate phylogeny and dates of origin while accounting for the possibility of rate heterogeneity across branches.

The colubroid snakes are a diverse (>2500 species), globally distributed group (Lawson et al., 2005) which date to the early Cenozoic (Burbrink and Pyron, 2008). Of the several NW representatives of the group (Natricinae, Crotalinae, Elapinae, Colubrinae, and Xenodontinae), the colubrine tribe Lampropeltini is one of the most conspicuous and well-studied (Williams, 1978; Rodríguez-Robles and de Jesús-Escobar, 1999). The lampropeltinines (rat, corn, and fox [*Pantherophis*, *Bogertophis*, and *Pseudelaphe*], king and milk [*Lampropeltis*], short-tailed [*Stilosoma*], bull, gopher, and pine [*Pituophis*], glossy [*Arizona*], scarlet [*Cemophora*] and long-nosed [*Rhinocheilus*] snakes) are common constrictors, distributed from Canada to Ecuador (Williams, 1978; Conant and Collins, 1998; Stebbins, 2003). Several recent studies have found that the Lampropeltini form a monophyletic clade endemic to the NW, thus rendering the cosmopolitan genus *Elaphe* paraphyletic (Rodríguez-Robles and de Jesús-Escobar, 1999; Utiger et al., 2002; Burbrink and Lawson, 2007).

Based primarily on trees inferred using mitochondrial evidence, the taxonomy of the group is in a state of flux and the monophyly of several genera (i.e. *Pantherophis*, *Pituophis*, and *Lampropeltis*) has been disputed, including the erection of a new genus (*Mintonius*) for the fox snakes (*Pantherophis vulpinus*; Bryson et al., 2007; Burbrink and Lawson, 2007; Collins and Taggart, 2008). Additionally, while many phylogeographic studies have used mtDNA to investigate biogeographic structure (Burbrink et al., 2000; Burbrink, 2002; Mulcahy, 2008; Rodríguez-Robles and de Jesús-Escobar, 2000), higher-level phylogenies based solely or primarily on mitochondrial data have not been well-supported (Rodríguez-Robles and de Jesús-Escobar, 1999; Burbrink and Lawson, 2007). Thus, multiple independent loci are desirable to infer phylogenies and

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estimate tree-based quantities such as divergence times (i.e. Wiens et al., 2008). Here, we present a phylogeny based on three nuclear genes (3368 bp), two of which are newly presented in this study, and six mitochondrial genes (4926 bp). We included representatives from all 31 of the traditionally described species of lampropeltinine. We use this phylogeny to address hypotheses regarding the timing of origin and diversification of the lampropeltinines, as well as generate a revised taxonomy of the group.

2. Methods and materials

2.1. Specimen acquisition and DNA sequencing

We collected tissue samples and DNA extracts for the 31 commonly recognized species of lampropeltinines (Conant and Collins, 1998; Rodríguez-Robles and de Jesús-Escobar, 1999; Stebbins, 2003) and two outgroups (*Coronella austriaca* and *Rhinechis scalaris*; Burbrink and Lawson, 2007). Additional sequence data was obtained only from existing datasets for the additional outgroup taxa *Gonyosoma oxycephalum* and *Elaphe carinata* (Utiger et al., 2002; Burbrink and Lawson, 2007). In addition to the new markers sequenced specifically for this study (SPTBN1; Vimentin Introns 4 and 5), we combined the nuclear and mitochondrial datasets of Rodríguez-Robles and de Jesús-Escobar (1999, 2000), Utiger et al. (2002) and Burbrink and Lawson (2007), which yielded six mitochondrial (mtDNA) loci (cyt-*b*, COI, ND1, ND2, ND4, and 12S) and one single copy (scnDNA) nuclear locus (c-*mos*). Genomic DNA was extracted using Qiagen DNEasy kits (Qiagen Corp.). Taxa which were missing any of the above genes were sequenced using the primers listed in the given references.

In addition, we sequenced three single copy, non protein-coding nuclear fragments: SPTBN1 (Mathhee et al., 2001) and Vimentin Introns 4 and 5 (Zehner and Paterson, 1983). We developed intron-spanning primer sets for SPTBN1 (Mathhee et al., 2001) and Vimentin Introns 4 and 5 (Zehner and Paterson, 1983) by comparing homologous regions from GenBank, and by sequencing taxa using the degenerate primers used by the original authors. These fragments were chosen based on two criteria: (i) existing as single copy in other tetrapods, with a (ii) reasonable amount of reported inter-specific divergence in other groups, suggesting that evolutionary rates would be high enough to yield phylogenetic signal at recent time scales. The following primer sets were used for amplification and sequencing: SPTBN1: SPTBN1SeqF 5′-ATA CAG GCT GAG CGA GTG AGA-3′, SPTBN1 SeqR 5′-AGC TGA CAT AGC TCT TGG TAA CA-3′; Vimentin Intron 4: VimExon4SeqF 5′-AAG CCC AAA TCC AGG ATC A-3′, VimIntron5R 5′-AGC ATA AGG GAG GAC ATA AAA-3′; Vimentin Intron 5: VimExon5F 5′-AAC AAT GAT GCC CTG CGC CA-3′, VimExon6R 5′-CAA TAT CAA GAG CCA TCT TTA CAT T-3′.

Gene fragments were amplified by polymerase chain reaction (PCR) using GoTaq Green Master Mix (Promega Corp.) according to the manufacturer's protocol. The existing genes (12S, c-mos, cyt-*b*, COI, ND1, ND2, and ND4) were amplified and sequenced at the annealing temperatures given in the original references. The annealing temperature used for amplifying and sequencing with the SPTBN1 primers was 51 degrees; for Vimentin Intron 4, 54 degrees; and for Vimentin Intron 5, 49 degrees, with an extension time of 90 seconds. Reaction products were cleaned using EXO-SapIT (USB Corp., 1 μl per 10 μl product) and sequenced using Dye Terminator Cycle Sequencing reaction chemistry (Beckman–Coulter Corp.) according to the manufacturer's protocol at the annealing temperatures noted above. Fragments were analyzed on a Beckman–Coulter CEQ-8000 automated sequencer. Protein coding segments (c-mos, cyt-*b*, COI, ND1, ND2, and ND4) were edited and aligned by eye in the program Sequencher (Genecodes Corp.) after confirming an absence of stop codons which would have suggested a pseudogene. Intron fragments and 12S were aligned using the MUSCLE algorithm (Edgar, 2004) implemented in the program Geneious (Biomatters Ltd.) using the default parameters.

2.2. Phylogenetic inference

To test hypotheses regarding the timing of diversification within the Lampropeltini, we inferred phylogenies using both Maximum Likelihood (ML) and Bayesian inference (BI) methods. For BI and ML analyses, we conducted mixed-, partitioned-model analyses on the concatenated data, partitioning by gene and by codon position (where applicable). The best fit evolutionary model for each gene was chosen by AIC in the program MrModeltest2 (Nylander, 2004). These models were as follows: HKY (*c*-mos), HKY + Γ (SPTBN1), HKY + Γ + I (COI), GTR + Γ (Vimentin Introns 4 and 5), and GTR + Γ + I (12S, cyt-*b*¸ND1, ND2, ND4). The appropriate model was then applied to each partition in the concatenated dataset. Both BI tree inference and divergence time estimation were performed using the program BEASTv1.4.8 (Drummond et al., 2006; Drummond and Rambaut, 2007). The analysis was run for fifteen million generations, with the first 2.5 million discarded as burnin. Stationarity was assumed when the effective sample size (ESS) reached >200 for all parameters (as per Drummond et al., 2006), which occurred prior to 2.5 million generations. The analyses were repeated multiple times to ensure that the chains were independently converging and not merely sampling local optima. The ML analyses were performed using the program RAxMLv7.0.3 (Stamatakis, 2006) under the same partitioning scheme, though RAxML only allows the use of the GTR model, which was enforced with the addition of Γ-distributed rate heterogeneity (GTRGAMMA). Support was assessed by performing one thousand non-parametric bootstrap (BS) replicates of the topology. To examine the potential impact of nuclear/mitochondrial gene tree incongruence (i.e. Sota and Vogler, 2001), we also repeated these analyses on the individual mtDNA and scnDNA datasets separately.

2.3. Divergence time estimation

To test temporal and evolutionary hypotheses regarding species richness, we estimated divergence times using the relaxed clock method of Drummond et al. (2006), implemented in the program BEASTv1.4.8 (Drummond and Rambaut, 2007). The data were partitioned by gene and given the same models used in the tree inference. The conditions of the analyses are identical to those of the phylogenetic inference. Rates and times were estimated under the uncorrelated lognormal tree prior, with a birth–death prior on speciation and Jeffrey's priors on the substitution model parameters. Five fossil constraints taken from the paleoherpetological literature which putatively represent extinct progenitors of modern taxa were placed on the tree. Using a lognormal distribution, we enforced fossil constraints in the most conservative manner possible, interpreting the age of the fossil as the mean of a time horizon during which an internal bifurcation took place, with the 95% prior distribution representing soft bounds on the divergence time of that node (*sensu* Yang and Rannala, 2006). Fossil information was taken from Holman (2000) as follows:

(a) The root of the tree (*Gonyosoma oxycephalum*) was given a prior credible interval (PCI) of 17.8–57.6 Ma, with a mean of 32 Ma, based on the oldest known colubrine fossil *Texasophis galbreathi* from the Orellan of the Oligocene.

(b) The MRCA of the Lampropeltini was given a mean date of 20.6 Ma (PCI = 11.4–37.1 Ma) based on the oldest known putative lampropeltinine, *Pseudocemophora cf. antiqua* from the late Arikareean of the Miocene.
(c) The divergence between the rat snakes (*Pantherophis*) and the pine snakes (*Pituophis*) was given a mean date of 15.5 Ma (PCI = 9.5–25.3 Ma) based on the oldest known rat snake, *Elaphe* (*Pantherophis*) *kansensis* from the early Barstovian of the Miocene.
(d) The divergence between the genera *Lampropeltis* and *Cemophora* was given a mean date of 13.75 Ma (PCI = 8.4–24.4 Ma) based on the oldest known kingsnake, *Lampropeltis similis*, from the medial Barstovian of the Miocene.
(e) The divergence between the sister taxa *Lampropeltis getula* and *Stilosoma extenuatum* was given a mean date of 6.8 Ma (PCI = 4.75–9.94 Ma) based on the oldest known fossils of *L. getula* and *Stilosoma vetustum* from the middle Hemphillian of the Miocene.

3. Results

3.1. Phylogenetic analysis

The combination of sequences added in this study and the existing Genbank data resulted in a concatenated alignment with a length of 8294 bp (3368 scnDNA, 4926 mtDNA) of data per species. The new sequences have been deposited on Genbank (Appendix S1) and TreeBase (S2278, M4334). After minor trimming for quality, the lengths of the new nuclear intron alignments were as follows: SPTBN1, 1106 bp; Vimentin Intron 4, 1064 bp; and Vimentin Intron 5, 631 bp. With some exceptions, the results of our phylogenetic analysis are broadly similar to those of Burbrink and Lawson (2007). Preliminary analyses supported recognition of two taxa of previously indeterminate status, the scarlet kingsnake (*Lampropeltis elapsoides*; Collins, 1991) and the Baja California gopher snake (*Pituophis vertebralis*; Rodríguez-Robles and de Jesús-Escobar, 2000; Stebbins, 2003), which were subsequently included in the analyses as distinct terminals (Fig. 1). The Lampropeltini are found to be monophyletic, with the OW *Coronella austriaca* the sister to the lampropeltinines (Fig. 1). Most internal relationships are highly supported by both Bayesian posterior probability (>0.95) and bootstrap proportion (BS > 0.70).

Though not fully resolved, the results from the nuclear genes alone are broadly concordant with the combined analyses (Fig. 1) and the mtDNA-only analysis. All terminal species are differentiable based on the scnDNA analyses alone. While the mtDNA-only analysis yields weak support for paraphyly of the rat, corn, and fox snakes (*Pantherophis*, including *Mintonius*) with respect to the pine, bull, and gopher snakes (*Pituophis*), which is the result presented in Burbrink and Lawson (2007), both the combined mtDNA + scnDNA and separate scnDNA analyses provide strong support for the reciprocal monophyly of those genera (Fig. 1). The scnDNA analyses yield well-supported sister relationships between *Pi. melanoleucus* and *Pi. ruthveni*, and between *Pi. catenifer* and *Pi. vertebralis*, a different result from the combined analyses.

Several phylogeographic lineages which had been elevated to species status using mtDNA alone are found here to be divergent based solely on nuclear evidence (i.e. *Pantherophis obsoletus* complex, *Pa. guttatus* complex, *Pituophis melanoleucus* complex; Burbrink et al., 2000; Burbrink, 2002; Rodríguez-Robles and de Jesús-Escobar, 2000). The scnDNA and mtDNA are also in concordance for other major features, such as the placement of *Senticolis* and corroboration of Bryson et al. (2007) in finding that *Stilosoma extenuatum* is sister to *Lampropeltis getula*. The scnDNA suggest a slightly different arrangement of the genus *Lampropeltis* (including a sister relationship between *L. calligaster* and *L. triangulum*, and between *L. alterna* and *L. mexicana*), though this was not well-supported. In light of the potential polyphyly suggested by Bryson et al. (2007), further research may be necessary to fully resolve relationships in this genus.

3.2. Divergence time estimation

The Lampropeltini diverged from OW relatives (*Coronella*) approximately 24.4 (19.0–30.5) Ma, and share a NW ancestor approximately 22.9 (17.9–28.8) Ma (Fig. 2). These results are broadly consistent with those of Burbrink and Lawson (2007), supporting the hypothesis of a trans-Beringian dispersal into the NW during the late Oligocene or early Miocene, as this was the only viable route during that time period. The lampropeltinines appear to have diversified primarily during the Miocene, with divergences between extant species occurring primarily during the late Miocene and Pliocene (Fig. 2). Unlike patterns reported for groups such as birds (Johnson and Cicero, 2004), the majority of terminal speciation events in the Lampropeltini predate the Pleistocene. Additionally, the diversification of all genera occurred in the mid- or early-Miocene (Fig. 2).

4. Discussion

4.1. Historical origins and diversification

Diversification of the modern genera and most species in the Lampropeltini occurred during the mid-Miocene and early Pliocene (Fig. 2). All diversification events at the generic level occurred in the early or mid-Miocene, and the majority of speciation events predate the Pleistocene (Fig. 2). Of the 31 currently described extant species, nine originated during the Miocene, with error estimates from the 95% Highest Posterior Density (HPD) rejecting a younger Pliocene origin (Fig. 2). Thirteen extant species originated during the Pliocene, and an additional seven occur near the Miocene/Pliocene boundary, which included the Pliocene in the 95% HPD (Fig. 2). Two terminal species pairs, *Pantherophis emoryi/slowinskii* and *P. alleghaniensis/spiloides* cannot reject an early Pleistocene origin in the lower tail of the 95% HPD. Only *P. alleghaniensis/spiloides* are estimated to have diverged after the Pliocene/Pleistocene boundary (1.7 Ma; Fig. 2).

These dates are consistent with the fossil record, which indicates that many extant species were present by the late Miocene (reviewed in Holman, 2000). As the majority of the lampropeltinine species occur in temperate NA (~25 species; Conant and Collins, 1998; Stebbins, 2003), this indicates that diversification in this group was not heavily influenced by Pleistocene glacial cycles. Diversification in the group was coincident with the mid-Miocene climatic optimum (~15 Ma), and continued during the formation of the Northern Hemisphere ice sheets (Zachos et al., 2001). Along with some divergence dating studies on birds, our results provide an additional piece of evidence suggesting that not all major radiations of vertebrate taxa are attributable to recent (i.e. Pleistocene) climatic cycles, at least in NA (i.e. Zink and Slowinski, 1995; Klicka and Zink, 1997). In contrast to some studies on organisms such as birds (i.e. Johnson and Cicero, 2004), the Pleistocene 'species pump' does not appear to be the driving force behind diversification in the Lampropeltini.

In terms of the degree and direction of diversification with respect to morphological and ecological specialization, the greatest amount of diversity appears early in the history of the Lampropeltini. For instance, the splits between the smaller king snakes and milk snakes and the larger rat and pine occurred during the Mio-

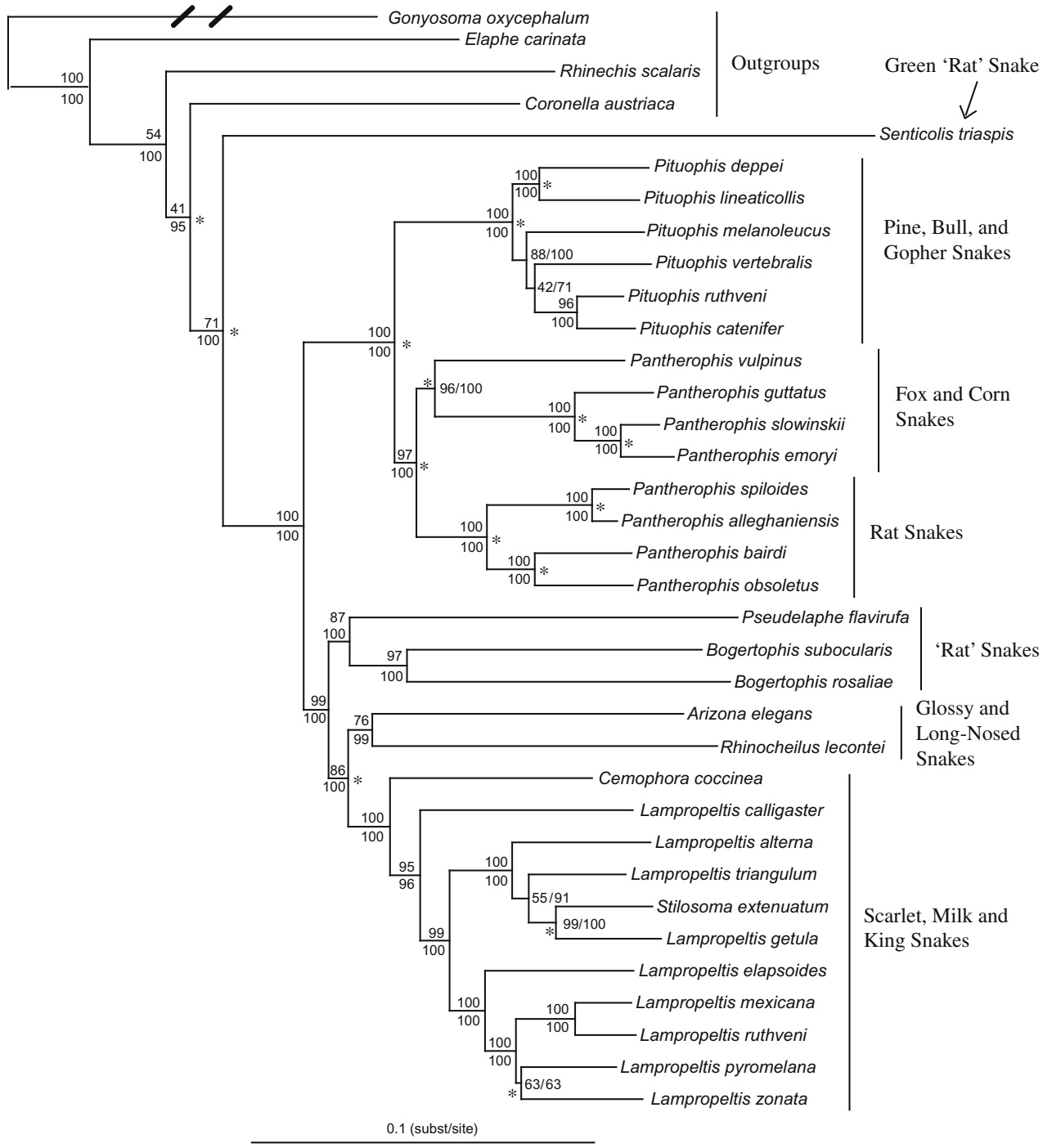

Fig. 1. ML phylogeny of the lampropeltinines, based on 8294 bp of nuclear and mitochondrial DNA, inferred using partitioned-model analysis in the program RAxML (Stamatakis, 2006). Values above branches represent non-parametric bootstrap proportions from 1000 replicates, values below branches represent Bayesian posterior probabilities from the program BEASTv1.4.8 (Drummond and Rambaut, 2007). Asterisks (*) indicate that the node was recovered in the most likely topology from the scnDNA analyses, regardless of support value.

cene (~19 Ma), while the more recent phylogeographic divergences exhibit less apparent ecological and morphological differentiation. In contrast, however, some relatively young sister species (e.g. *Lampropeltis getula* and *Stilosoma extenuatum*, ~6 Ma) exhibit a great degree of morphological specialization (Conant and Collins, 1998), and other species such as the milk snake (*Lampropeltis triangulum*) exhibit large amounts of ecological and morphological diversity within a putative single species (Williams, 1978). Thus, the underlying relationships between the temporal aspects of phylogenetic diversification, morphological variation, and ecological specialization require further study.

4.2. Improved phylogenetic resolution

The use of multiple nuclear and mitochondrial genes and total sampling of the recognized species diversity has greatly improved resolution of the phylogeny of the lampropeltinines over previous efforts (Rodríguez-Robles and de Jesús-Escobar, 1999; Burbrink

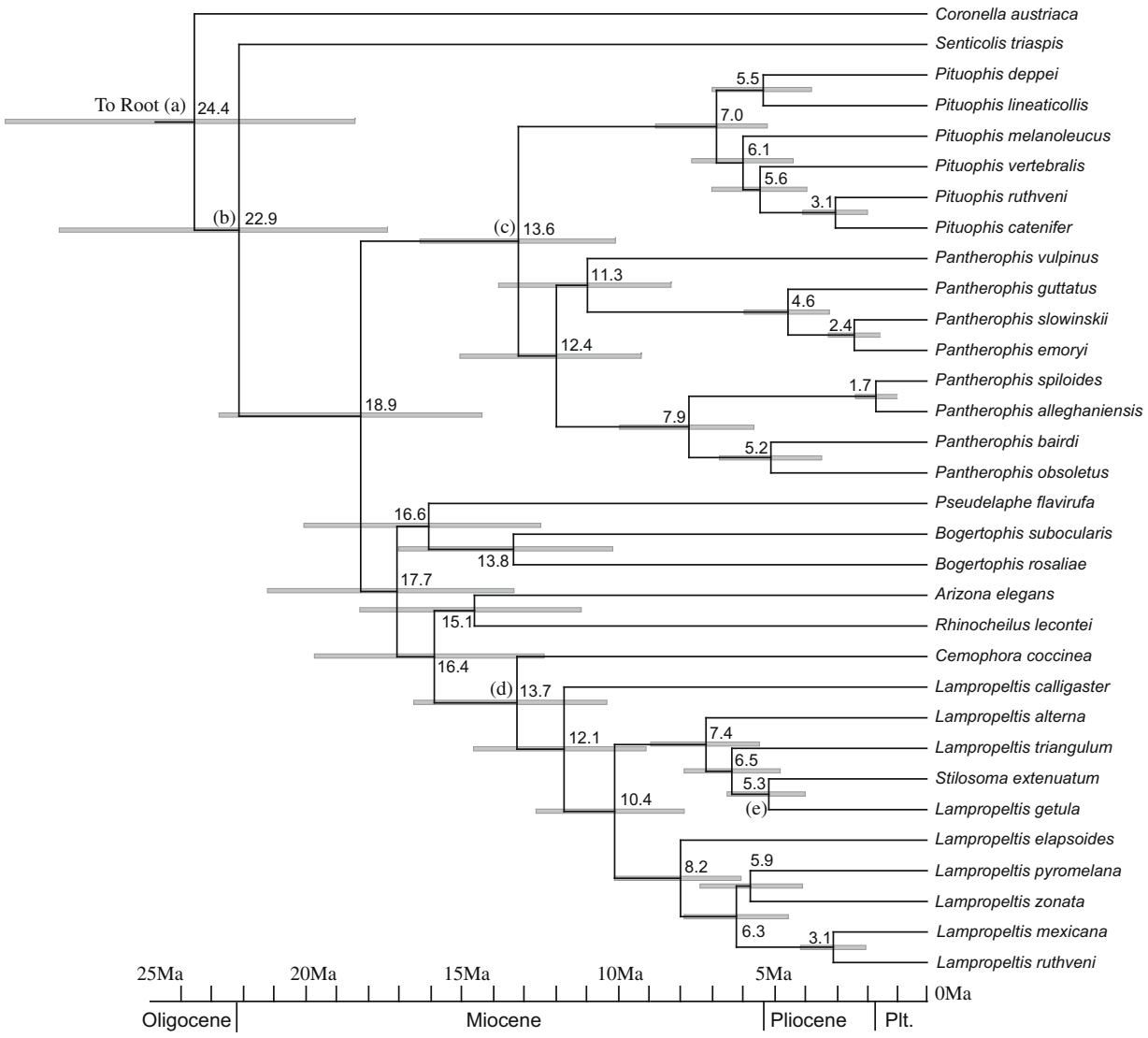

Fig. 2. Bayesian inference chronogram of the Lampropeltini inferred using the program BEASTv1.4.8 (Drummond and Rambaut, 2007). Values at nodes represent the mean of 12.5 million post-burnin generations, bars indicate the 95% Highest Posterior Density. Topology has been truncated to show only the immediate outgroup *Coronella austriaca*. 'Plt.' refers to the Pleistocene. Letters in parentheses at nodes correspond to the listed fossil constraints.

and Lawson, 2007). We find strong support for the monophyly of the rat snakes (*Pantherophis*) and the pine snakes (*Pituophis*), as well as for the placement of the monotypic genera *Arizona*, *Cemophora*, *Rhinocheilus*, *Senticolis*, *Pseudelaphe*, and *Stilosoma*. In addition, we find that many phylogeographic lineages which were elevated to species based on mtDNA (*Pi. melanoleucus*, *Pi. catenifer*, *Pi. ruthveni*; *Pa. obsoletus*, *Pa. alleghaniensis*, *Pa. spiloides*, *Pa. bairdi*; *Pa. guttatus*, *Pa. emoryi*, *Pa. slowinskii*) are also well differentiated by nuclear evidence alone, though we did not examine population variation to ensure reciprocal monophyly. This suggests that mtDNA markers are likely sufficient for tracking phylogeographic structure and identifying species in many cases (Zink and Barrowclough, 2008). Despite this, we still recommend that researchers attempt to corroborate mtDNA phylogeographic estimates with nuclear markers. The other caveat implied by these findings is that species diversity in the Lampropeltini, especially potentially cryptic phylogeographic lineages, may be even greater than the currently recognized species which we have sampled here. In particular, the milksnake (*Lampropeltis triangulum*) ranges from Canada to Ecuador and consists of more than 20 subspecies (Williams, 1978), suggesting a strong potential for further undescribed specific diversity.

4.3. Notes on taxonomy

Based on the results of this study (Fig. 1; Appendix S1), some discussion of taxonomic nomenclature is necessary to accurately reflect historical evolutionary relationships within the Lampropeltini, and to promote greater understanding of current diversity. With regard to the clade consisting of the rat and pine snakes and relatives, the taxonomic conclusions of Burbrink and Lawson (2007) and Collins and Taggart (2008) are shown to be inaccurate. The monophyletic genus *Pantherophis* (Fitzinger, 1843) is restored for the fox, rat, and corn snakes (*Pa. alleghaniensis*, *Pa. bairdi*, *Pa. emoryi*, *Pa. guttatus*, *Pa. obsoletus*, *Pa. spiloides*, and *Pa. slowinskii*), while the monophyletic *Pituophis* (Holbrook, 1842) is preserved for the pine snakes (*Pi. melanoleucus*, *Pi. catenifer*, *Pi. deppei*, *Pi. lineaticollis*, *Pi. ruthveni*, *Pi. vertebralis*). The genus *Mintonius* (Collins and Taggart, 2008) is now considered a junior synonym of *Pantherophis*. We have placed the genus *Stilosoma* (Brown, 1890) into synonymy with *Lampropeltis* (Fitzinger, 1843), rendering the emendation of the sole species from the erstwhile genus *L. extenuata* (short-tailed king snake). We find that *L. elapsoides* is well differentiated from *L.*

triangulum (Collins, 1991), and that *Pi. vertebralis* is distinct from *Pi. catenifer* (Rodríguez-Robles and de Jesús-Escobar, 2000; Stebbins, 2003). The continued recognition of the genera, *Arizona*, *Bogertophis*, *Cemophora*, *Pseudelaphe*, *Rhinocheilus*, and *Senticolis* is warranted since they do not render any of the other genera within the Lampropeltini paraphyletic.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.02.008.

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