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Speciation at the Mogollon Rim in the Arizona Mountain Kingsnake (*Lampropeltis pyromelana*)

Frank T. Burbrink [a,b,*], Helen Yao [c], Matthew Ingrasci [d], Robert W. Bryson Jr. [e], Timothy J Guiher [b], Sara Ruane [b]

[a] Biology Department 6S-143, 2800 Victory Blvd., College of Staten Island/CUNY, Staten Island, NY 10314, United States
[b] Biology Doctoral Program, City University of New York, Graduate Center 365 Fifth Avenue, New York, NY 10016-4309, United States
[c] Science, Math and Engineering, Staten Island Technical High School, 485 Clawson Street, Staten Island, NY 10306, United States
[d] University of Texas at Arlington, Biology Department, Box 19498, Arlington, TX 76019-0498, United States
[e] Ecology and Evolutionary Biology Section, School of Life Sciences, University of Nevada, Las Vegas, 4505 Maryland Parkway Las Vegas, Nevada 89154-4004, United States

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ABSTRACT

Studies of speciation and taxon delimitation are usually decoupled. Combining these methods provides a stronger theoretical ground for recognizing new taxa and understanding processes of speciation. Using coalescent methods, we examine speciation, post-speciation population demographics, and taxon delimitation in the Arizona Mountain Kingsnake (*Lampropeltis pyromelana*), a species restricted to high elevations in southwestern United States and northern Mexico (SW). These methods provide a solid foundation for understanding how biogeographic barriers operate at the regional scale in the SW. Bayesian species delimitation methods, using three loci from samples of *L. pyromelana* taken throughout their range, show strong support for the existence of two species that are separated by low elevation habitats found between the Colorado Plateau/ Mogollon Rim and the Sierra Madre Occidental. Our results suggest an allopatric mode of speciation given the near absence of gene flow over time, which resulted in two lineages of unequal population sizes. Speciation likely occurred prior to the Pleistocene, during the aridification of the SW and/or the uplift of the Colorado Plateau, and while these species occupy similar high-elevation niches, they are isolated by xeric conditions found in the intervening low deserts. Furthermore, post-speciation demographics suggest that populations of both lineages were not negatively impacted by climate change throughout the Pleistocene. Finally, our results suggest that at least for this group, where divergence is old and gene flow is low, Bayesian species delimitation performs well.

1. Introduction

The causes of speciation have been investigated in numerous organisms inhabiting diverse regions of the Earth. Evolutionary biologists have defined, modeled and studied the possible modes of speciation over the last century, and since the Modern Synthesis, advances in computational methods have helped determine if general processes drive the formation of lineages (Coyne and Orr, 2004; Hey, 2009; Mayr, 1942, 1963). Diversification likely occurs along a geographic spectrum, where on one end allopatric speciation is marked by having no gene flow due to a physical barrier, and on the other end sympatric speciation shows a complete lack of geographic isolation (Bolnick and Fitzpatrick, 2007; Gavrilets, 2004; Mayr, 1963; Nosil, 2008). Of the many possible avenues of diversification, allopatric speciation is one of the most widely cited (Coyne and Orr, 2004; Wiens and Graham, 2005) and is commonly implicated in many phylogeographic studies (Pyron and Burbrink, 2010).

While divergence between species often happens in isolation with no gene flow, one alternative, parapatric speciation, suggests that low levels of gene flow may occur as populations diverge (Coyne and Orr, 2004; Hey, 2006; Nosil, 2008). Although it is expected that divergence in the face of gene flow is generally unlikely to occur, numerous examples have demonstrated the plausibility of the phenomenon (Hey, 2006; Niemiller et al., 2008). Alternatively, populations may diverge allopatrically, but recurrent gene flow from subsequent changes in geographic barriers and climate may create the illusion of parapatric speciation. Estimating the timing of migration (m) can help differentiate between allopatric speciation with secondary gene flow (m = late) from parapatric speciation (m = early; Slatkin, 1989; Slatkin and Maddison, 1989; Won and Hey, 2005), but changes in m and population structure through time can obscure these patterns. Finally, peripatric speciation, where differences in effective population size (N_e) may occur at the initial split of lineages is less likely to happen given that genetic drift would have to break up coadapted ancestral gene

* Corresponding author.
E-mail address: frank.burbrink@csi.cuny.edu (F.T. Burbrink).

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complexes in order for intrinsic reproductive isolation to occur (Losos and Glor, 2003; Mayr, 1954; Templeton, 1980).

The first step to studying the geographic context in which speciation occurs usually requires the identification of independently evolving lineages. Phylogeographic methods have often relied on identifying species using single gene-tree estimates or previous morphological designations of taxa (Avise, 2000; Edwards, 2009). For most phylogeographic studies, identifying patterns of phylogeographic structure takes precedence over understanding the processes that gave rise to those patterns (Edwards and Bensch, 2009; Zink and Barrowclough, 2008). However, the application of coalescent methods using multiple unlinked genes can aid in both delineating species and understanding the processes of speciation (Knowles and Carstens, 2007; Yang and Rannala, 2010). Going beyond the standard gene-tree paradigm, these methods assess species-tree uncertainty due to the coalescent using multiple gene trees to identify independently evolving lineages. These new methods offer a substantial improvement over traditional gene-tree techniques because they account for lineage sorting processes due to N_e size and assess the probability of speciation in a quantifiable manner (Carstens and Knowles, 2007; Leaché and Fujita, 2010; Yang and Rannala, 2010). At least for newly arisen taxa, coalescent species-delimitation techniques can be used in conjunction with spatial information to identify biogeographic barriers responsible for lineage divergence. Moreover, when augmented with other coalescent tests of migration between lineages, divergence time, and changes in N_e, a clear picture can be constructed about the process of speciation and post-divergence dynamics of population demographic changes in relationship to the environment (Heled and Drummond, 2008; Hey, 2006; Hey and Nielsen, 2004). With respect to lineage demographics that follow divergence, populations may expand as new habitat is colonized, or alternatively, shrink as habitable area decreases (Burbrink and Castoe, 2009; Hewitt, 2000). Therefore, the trajectories of N_e could be quite different between newly formed species given the geological history of the areas occupied by the lineages and the initial sizes of new species.

Phylogeographic structure has been examined in numerous organisms that inhabit the southwestern deserts and associated montane regions of North America (SW). These areas have been shown to yield deep phylogeographic structure in a wide range of organisms, including plants, insects and vertebrates (Barber, 1999; Bryson et al., 2010, 2011; Castoe et al., 2007; Devitt, 2006; Hafner and Riddle, 2008; McGuire et al., 2007; Mulcahy, 2008; Pyron and Burbrink, 2009a; Riddle and Hafner, 2006). This area is interesting to phylogeographers because of the wide variety of habitat changes associated with temperature, precipitation, and topography. Separation among deserts, sky islands and mountain ranges, as well as changes in climate, have been implicated in the formation of species, which occurred as early as the Miocene and as late as the Pleistocene (Barber, 1999; Downie, 2004; Lomolino et al., 1989; Masta, 2000; McCormack et al., 2010). However, for many organisms with clear phylogeographic structure in this region, tests have not been performed to elucidate modes of diversification, levels of gene flow, or historical population demography.

Within the SW, areas of high-elevation habitats are sharply isolated by the intervening low desert habitats. These highlands mainly include the Colorado Plateau north of the Mogollon Rim in Arizona and New Mexico, isolated Madrean sky islands and the continuous range of mountains along the Sierra Madre Occidental in Mexico (Lomolino et al., 1989; Stevens and Polhemus, 2008). These mesic areas of high elevation from Utah to central Mexico are separated by low-elevation desert habitats at the interface of the Chihuahuan and Sonoran Deserts in southeastern Arizona and southwestern New Mexico (Barber, 1999; Lomolino et al., 1989; Masta, 2000). Although not directly impacted by glaciers, these highlands have been affected by climate changes throughout the Pleistocene. Cooler mesic montane habitats sink to the valleys during glacial maxima, and then recede to higher elevations during warmer interglacials. Globally, these glacial processes have impacted diversification as well as changes in population size through time in several organisms (Betancourt, 1990; Comes and Kadereit, 1998; Ditto and Frey, 2007; Hewitt, 2000, 1996). Across the SW, however, some taxa have highly structured lineages that formed prior to major Pleistocene climate changes (Barber, 1999; Bryson et al., 2010; Masta, 2000; Smith and Farrell, 2005), whereas other species show little to no population structure currently (Downie, 2004).

Snakes have been used in a wide variety of studies to examine the impacts of biogeographic barriers and climate on lineage formation and population demography (Burbrink and Castoe, 2009; Burbrink et al., 2008; Devitt, 2006; Pyron and Burbrink, 2009a). Here, we address mechanisms of speciation in the brightly colored Arizona Mountain Kingsnake (*L. pyromelana*), an organism absent from low desert habitats. This snake is found at high elevations (1400–2734 m) intermittently from Utah to northwestern Mexico (Fig. 1; Ernst and Ernst, 2003; Tanner et al., 1982; Tanner, 1953). At lower elevations they occur in chaparral and piñon-juniper pine-oak, and at higher elevations these snakes are found in evergreen woodlands. Because of its particular habitat requirements and fragmented distribution, this species should be useful for investigating the effects of biogeographic barriers and climate on lineage formation and population demography.

Using three independent loci, we examine phylogeographic structure in the Arizona Mountain Kingsnake to address several hypotheses. First, is population structure of this high-elevation snake influenced by uninhabitable low elevation habitat? If so, this prediction would suggest that distinct lineages should be found on the separate areas of high elevation (i.e., the Colorado Plateau, the Madrean sky islands and the Sierra Madre Occidental). Phylogeographic structure has been found on each of these areas of high elevation in a variety of organisms (Barrowclough et al., 2006; Goldberg et al., 2004; Haenel, 2007; Lamb et al., 1997). Second, using coalescent species delimitation models, we ask if the three unlinked loci provide support for the existence of more than one species. Using gene trees and estimates of N_e and timing of divergence, these models assess the probability that multiple species can be delineated (Yang and Rannala, 2010). Third, if multiple independent lineages exist, then when did divergence occur, and was it associated with major climatic changes during the Pleistocene? Fourth, did limited migration accompany speciation, or did these lineages form in strict allopatry? Fifth, did diversification produce lineages of equal population size, or is there evidence that indicates the newly formed species were of vastly different N_e sizes? Sixth, did the population sizes of distinct lineages fluctuate throughout the Pleistocene? Providing tests for all of these hypotheses allows us to understand modes of speciation, impacts of climate change, and diversity in the SW.

2. Methods and materials

2.1. Data collection

We obtained 45 samples of *L. pyromelana* collected throughout their range (Fig. 1; Appendix A) and used *Lampropeltis triangulum* as the outgroup (Pyron and Burbrink, 2009b). DNA was extracted using the Qiagen DNeasy kits (tissue protocol) to obtain genomic DNA from samples of shed skin, liver, muscle tissue or whole blood. Three loci (one mtDNA and two nDNA) were amplified using GoTaq Green MasterMix (Promega Corp.) according to the manufacturer's specifications, with a 90 s extension time. The

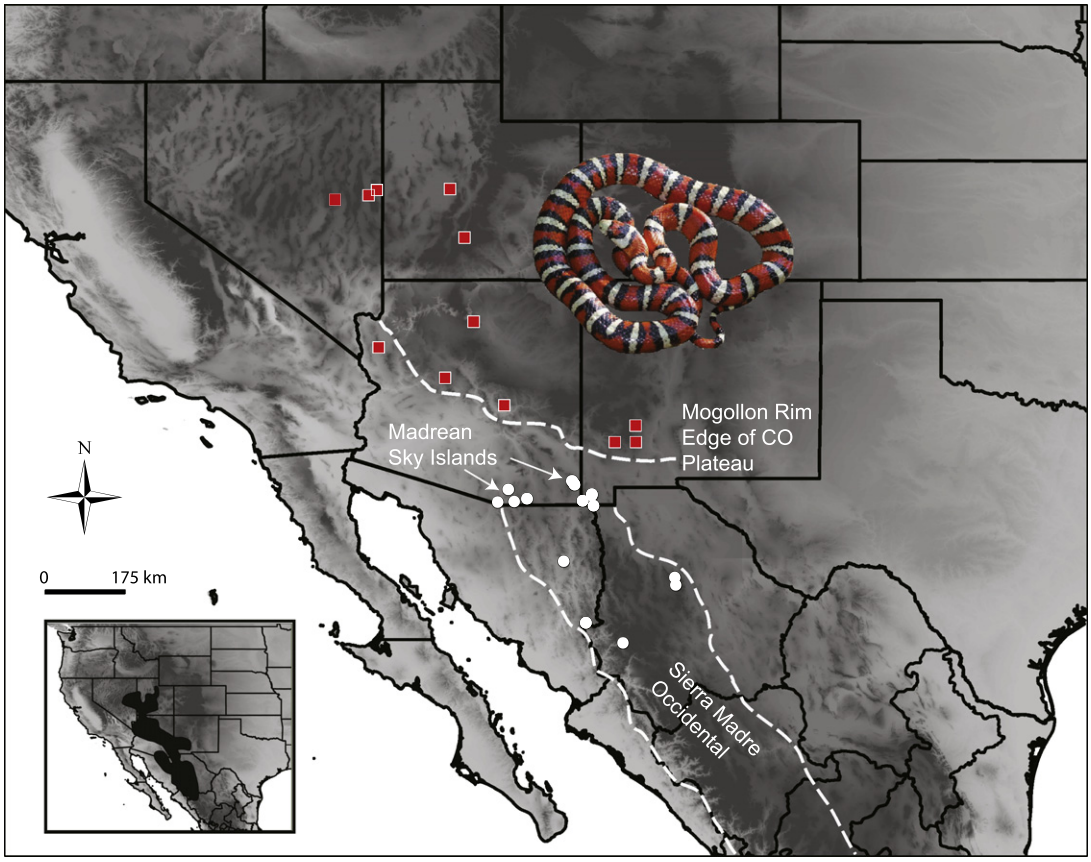

Fig. 1. Range of *Lampropeltis pyromelana* showing the northern Colorado Plateau (CP) clade and the Sierra Madre Occidental (SMO) clade as well as the edge of the Colorado Plateau, Madrean Sky Islands and range of the Sierra Madre Occidentals. Insert shows the known range of the species (Stebbins, 2003).

polymerase chain reaction (PCR) products were cleaned using 1 μL of ExoSap-IT (USB Corp.) per 10 μL of PCR product. The sequencing reaction consisted of 3 μL Beckman-Coulter DTCS, 2 μL primer (5 lm), 2 μL template and 3 μL deionized water. We used H14910 and THRSN2 primers (Burbrink et al., 2000) for PCR amplification and MxTriangF (5′-CGA TTC TTT GCC YTA CAC TT-3′) and MxTriangR (5′-GAC TGA TAT GGR TGG AAT GGA-3′) primers for the cycle sequencing reactions. For some particularly old or degraded templates, Cytb was amplified and sequenced in two fragments, using H14910 + MxTriangR and MxTriangF + THRSN2. Primers for the nuclear gene PRLR (nDNA; 585 bp) were amplified and sequenced using PRLR-F1 and PRLR-R3 (Townsend et al., 2008). We also sequenced a single anonymous locus 2CL8 (nDNA; 505 bp) developed using the protocol in Noonan and Yoder (2009). Amplification and sequencing of 2CL8 used the primers 2CL8F (5′-CCC TCA ATC TAG CCC AGT-3′) and 2CL8R (5′-GAT TAG CAG GAA ACT CT-3′). The protocol for amplification of 2CL8 was the same as Burbrink et al. (2000) but with an annealing temperature of 48 °C. No gaps were present in any of the genes among all samples and all sequences were aligned by eye using Sequencher 4.5 (Genecodes, 2000). For the two nuclear genes, the phase of heterozygous genotypes was resolved using Phase v2.1.1 (Stephens and Donnelly, 2003). On all sequences where two or more heterozygosities were present, Phase was run for 100 iterations using default values for all parameters, with a thinning interval of 1, and a burnin of 100. In order to check reliability of the results, five replications were performed for each gene with a different starting seed each time. Haplotype frequency estimates for each run were checked for consistency and phased genotypes were used for all coalescent analyses.

2.2. Phylogeographic inference

We used Bayesian inference (BI) and maximum likelihood (ML) to estimate phylogeographic structure using all loci. jModeltest (Posada, 2008) was used to estimate the most appropriate model of evolution for each gene using the BIC criterion. With the best available model, genes were partitioned and two runs were simultaneously conducted in MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001) using three heated and one cold chain for 2×10^7 generations. Convergence was assessed using Gelman and Rubin's *r* statistic (Gelman, 2004). Maximum likelihood (ML) analysis was performed using RAxMLv7.4.3 (Stamatakis, 2006) with this same dataset. The GTRGAMMA model was used for each gene partition, and 1000 nonparametric bootstrap replicates (Felsenstein, 2004) were performed to assess node support. Bayesian posterior probabilities (Pp) greater than 95% are considered strong support for a clade, while bootstrap proportions (BS) greater than 70% are considered adequate support (Felsenstein, 2004; Hillis and Bull, 1993).

Because phylogeographic structure may be dominated by the faster evolving mtDNA gene, we produced a genetic network using only the two nuclear genes. This network was inferred using SplitsTree 4.10 (Huson and Bryant, 2006) with the NeighborNet algorithm (Bryant and Moulton, 2004).

2.3. Species delimitation

We estimated the probability that the lineages with deep phylogeographic splits represent undiscovered species using two species-delimitation approaches. First, we used Structurama v2.2

(Huelsenbeck and Andolfatto, 2007) to infer the number of groups and to assign individuals to those groups following a Dirichlet process prior for K populations with the gamma shape and scale parameter set at 1.0 and 10.0, respectively. Using MCMC, we ran Structurama four times for 5×10^6 generations, saving every 100th sample, with a burnin of 10×10^5. The appropriate value of K was chosen as the one with the highest posterior probability value.

We also used the program Bayesian Phylogenetics and Phylogeography (BPPv2.0) (Yang and Rannala, 2010) to delimit species using the reversible-jump Markov chain Monte Carlo (rjMCMC) method. BPP estimates and uses Θ_a ($N_e \times \mu$ [mutation rate] for each species), τ_a (the time of origin for each species) and τ_d (the timing of diversification into two descendent species) to account for unknown gene trees and ancestral coalescent processes. This program infers the joint posterior distribution of species delimitation and species tree and yields a posterior probability associated with the existence of each species (e.g., a node separating two species). Using the entire phased dataset and coded by each gene we parameterized BPP in the following manner. First, we used a guide tree based on the designation of specimens into the two lineages found in the ML/BI trees (Fig. 2). These are both equivalent to designating specimens as originating on the (1) Colorado Plateau (CP) or (2) Sierra Madre Occidental and the Madrean sky islands (SMO). For these lineages to be considered distinct species under a general lineage species concept, the program assesses the probability of the node separating the two species exist under a general lineage species concept (de Queiroz, 2007) while accounting for lineage sorting due to time since divergence and population size. The current implementation of the program does not account for the potential source of error due to gene flow between lineages, therefore the impact of admixture on delimiting species is currently unknown. We used algorithm 0 with several values for the fine-tuning parameter, ε (5, 10, 15, 20), to ensure that the rjMCMC mixes properly among species-delimitation models. Similar to Leaché and Fujita (2010), we parameterized both ancestral population size (Θ) and root age (τ_o) using a gamma (Γ) distribution (a,b) for very large populations and deep divergences, Γ (1,10) and small ancestral and shallow divergences Γ (2, 2000). For each of these, we ran four analyses with different starting seeds for 1000,000 generations with a burnin of 10,000 and thinning every five generations. The swapping rates for each parameter ranged from 0.30 to 0.70. Finally, to test the robustness of these results, we repeated these runs where individuals in the analyses were randomized to either group.

2.4. Speciation process

To infer the timing of speciation, ancestral population sizes at speciation, and whether immigration occurred subsequent to speciation, we used the program IMa (Hey and Nielsen, 2007) on the full phased dataset. We first asked, could climatic cycles of the Pleistocene be implicated in the divergence of high-elevation lineages (see results) on the Colorado Plateau and Sierra Madre Occidental? Given credible intervals, divergences occurring prior to 1.8 Ma would suggest that Pleistocene climatic cycles were not relevant for speciation in this snake. Secondly, we asked whether the two lineages attained equal N_e sizes. Even though divergence could have occurred via allopatric mechanisms, populations may have been equal or very different. Finally, we asked if divergence was accompanied by gene flow.

The program IMa uses MCMC to estimate the joint posterior probability of six demographic patterns: N_{e1} (effective population size of lineage 1-CP), N_{e2} (effective population size of lineage 2-SMO), N_{ea} (ancestral effective population size), m_1 (migration of CR into SMO), m_2 (migration of SMO into CP) and τ (time of divergence). To scale time in number of years and N_e, we used a mtDNA (Cytb) substitution rate of 1.086×10^{-5} substitutions/locus/year

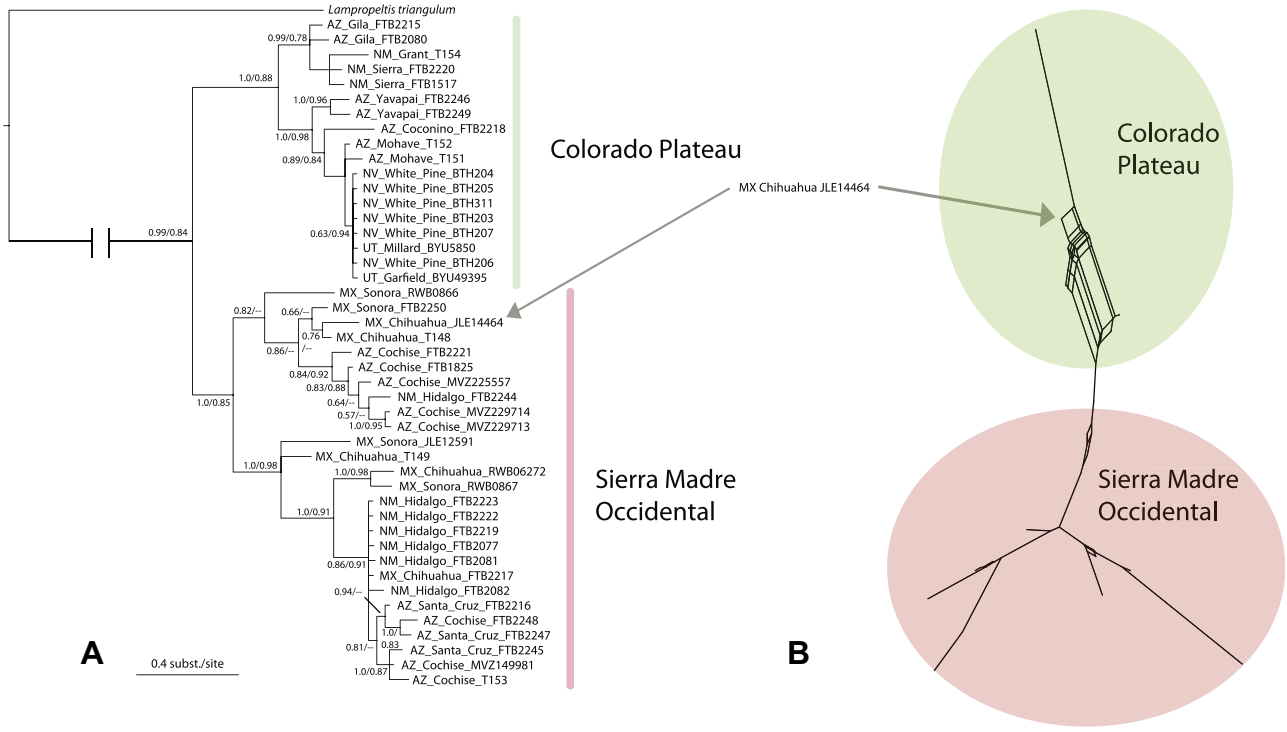

Fig. 2. Phylogeographic relationships of *L. pyromelana* populations showing (A) results from the ML (RAxML) analysis with substitution models partitioned among the three loci with posterior probability support (MrBayes) indicated above the slash and bootstrap support (RAxML) indicated below the slash and (B) a genetic network composed using the NeighborNet algorithm (SplitsTree) with only the two nuclear loci. Composition of the two major clades, Colorado Plateau and Sierra Madre Occidental/Madrean Sky Island, are identical between the two trees except the sample from Chihuahua, MX as indicated.

(95% HPD: 7.8×10^{-6}–1.4×10^{-5}) sites estimated for members of the genus *Lampropeltis* (see dataset from Pyron and Burbrink, 2009b) and a generational time of 2.5 years (estimated as an average from closely related members within the genus; *Lampropeltis getula*, *L. triangulum*, and *Lampropeltis calligaster*; Ernst and Ernst, 2003; Werler and Dixon, 2000). The HKY model (Hasegawa et al., 1985) was applied to each gene and the mtDNA locus used an inheritance scalar of 0.25 and the two nuclear loci = 1.0. The dataset was analyzed four times with different seeds in the MCMC to estimate the unscaled priors for Θ of each population q_1, q_2, m_1, m_2 and τ and adequate values for the geometric heating of 30 chains. We ran the final dataset three times visiting 10 million trees with the following priors: q_1, $q_2 = 10$, m_1, $m_2 = 1$, and $\tau_1 = 11$. Finally, from this output, log-likelihood ratio tests (2LLR) were used to examine the significance of a series of nested models in the "-L" mode of IMa. Given the five parameters of the model, all nested models are tested against the full model, where $N_{e1} \neq N_{e2} \neq N_{eA}$ and $m_1 \neq m_2$. Therefore, the 16 nested models test if some or all of these parameters are equivalent to the full model using the 2LLR test from a chi-square (or mixed) distribution. Additionally, m_1 and m_2 are evaluated where migration is effectively = 0.

2.5. Historical demography

We examine changes in demography over time using the method of extended Bayesian skyline plots (EBSP) as described and implemented in Beast 1.5.4 (Drummond and Rambaut, 2007; Heled and Drummond, 2008). We wanted to determine if lineages experienced population declines or expansions along high elevations on the CP and SMO following diversification. This method has the benefit of considering the coalescent history of each gene simultaneously to characterize N_e over time for each of the two lineages (CP and SMO). The time axis was scaled using the substitution rate for the Cytb gene (see above). All EBSP were run for the appropriate number of generations with a burnin of 10×10^6 to achieve reliable samples-size estimates (ESS > 200) as analyzed in Tracer v.1.4 (Drummond and Rambaut, 2007). We also determined the probable number of population size shifts in Tracer by examining the frequency distribution of these changes under the parameter demographic.populationSizeChanges.

We calculated a series of summary statistics on each gene that included sample size, number of haplotypes, π (nucleotide diversity), and Tajima's *D*, each calculated in DnaSP 5 (Librado and Rozas, 2009). We compared our results from the EBSP to Tajima's *D*, where significantly negative values either indicate population expansion or a selective sweep.

2.6. Ecological-niche modeling

The potential distributions of both lineages inferred in the phylogenetic analyses were estimated by generating ecological niche models (ENM) using a maximum entropy method in the program Maxent 3.3.3 (Phillips et al., 2006; Phillips and Dudik, 2008). The 19 bioclim variables describing temperature and precipitation from the WorldClim data set (Hijmans et al., 2005) at 30-s spatial resolution were used to construct ENM's. Models were trained using museum and published records in addition to georeferenced localities for all individuals used in the molecular analysis (Appendix A). This dataset was reduced by removing duplicate presence records and any museum samples that could not be confidently assigned to either lineage based on location, yielding 53 and 64 localities for the CP lineage and SMO lineage, respectively. We used auto features along with the default regularization multiplier (1.0). The number of iterations was increased to 5000 to allow the algorithm to run to the default convergence threshold (10^{-5}). We performed 10 replicate runs using a different random seed and subsampling, which randomly designated 75% of the samples for training and 25% of the samples for testing the model in each replicate. This provided an estimate of the sensitivity of the predicted distribution to the samples used to train and to test the model. Finally, predictions of the potential distribution for each lineage were constructed using all available samples to train the model. We evaluated model performance using two criteria, the threshold-independent receiver operating characteristic curve (AUC) and the threshold-dependent binomial omission tests. Sufficient discrimination between 'presence' and 'absence' is indicated by AUC values greater than 0.7 (Swets, 1988). Resulting distributions were projected in DIVA-GIS using the minimum training presence as the binary threshold.

Similar to other studies, we examined the possibility that the two lineages are separated by an area of unsuitable niche, which addresses the potential role for niche conservatism in the origin of the lineages (Wiens, 2004; Kozak and Wiens, 2006; Glor and Warren, 2011). Therefore, we performed a second analyses designed to examine the suitability of low elevation habitat for the two clades of *L. pyromelana*. Because allopatric speciation may be the cause of the formation of these distinct lineages, we assessed the predictability of areas potentially unoccupied by *L. pyromelana*. We also examined whether the areas suitable for each of the two lineages are significantly different. For the low elevation areas, we took 100 random samples below the documented range of this species (1400 m; Ernst and Ernst, 2003). Since bioclim variables may be autocorrelated, we omitted parameters that were correlated (Spearman rank) >0.90 (Kozak and Wiens, 2006; Pyron and Burbrink, 2009a; Rissler and Apodaca, 2007; Shepard and Burbrink, 2009), which ultimately yielded bioclim 1–5, 9, 12, 14, 15, 17 and 19 for further analyses. Taking the unique localities for each lineage and the low-elevation samples, we used principal-components analysis (PCA) with varimax rotation to reduce the intercorrelated bioclim parameters to a smaller number of independent variables in Statistica 9.1 (Statsoft, 2010). We retained principal components with eigenvalues >1 that explained >10% of the variation. Since PCA scores were not normally distributed, we used a multiple comparison Kruskall-Wallis ANOVA by ranks to determine whether each lineage and the low elevation samples were significantly different for each component, which would suggest that low areas have unsuitable habitat and that lineages may have diverged with respect to niche.

Additionally, using the 19 bioclim variables at 30 s spatial resolution, we determined if the predicted niche between the two lineages of *L. pyromelana* differed significantly using the "Warren's et al.'s *I*" and Schoener's *D* in the ENMTools 1.3 package (Schoener, 1968; Warren et al., 2008). Here, both *I* and *D* can range from 0, indicating no overlap between lineages, to 1, indicating identical niche. For the identity test, ENMTools assesses equivalency between ecological niche models by comparing the observed values of *D* and *I* for our empirical data with a distribution of values of *D* and *I* based on 100 randomized pseudoreplicates using a one-tailed test. The pseudoreplicate distribution is generated by randomly assigning occurrence points from both groups into one lineage or the other, simulating the potential overlap of a group of points occurring across a given geographical area (Warren et al., 2008). Niche identity is rejected if our empirical values of *I* and *D* are significantly lower than the distribution predicted from the randomized data sets. Since it is unlikely that niches for allopatric lineages are identical, we also used the background test in ENMtools. The test for background similarity assesses whether the two lineages are similar by chance using empirical values for *I* and Schoener's *D*. Using a 2-tailed test (because it is possible that empirical niches are more or less similar than the null) we determine if the ENM produced from one lineage using empirical points

is similar to the ENM for the other lineage generated from 100 random localities (randomly drawn from a pool of 1000 localities) taken from the range of the second lineage. This was conducted for both lineages in two separate tests.

3. Results

3.1. Phylogeographic structure and species delimitation

Sequences of *L. pyromelana* and *L. triangulum* were obtained and aligned by eye since no gaps or size differences were detected for Cytb (1117 bp), PRLR (585 bp), or 2CL8 (505 bp). All sequences have been deposited in Genbank under accession numbers JN034257-JN034381. Four individuals failed to yield DNA sequence for both nuclear genes (BYU 5850; BYU 49395; T-153 and FTB 2250), and these individuals were not used in coalescent analyses or to estimate parameters from Structurama. The number of variable sites (Table 1) was greatest for Cytb (98 sites), followed by 2CL8 (9 sites) and PRLR (7 sites). The most appropriate model of substitution for Cytb was GTR + Γ partitioned among the three codons, PRLR was HKY + Γ, and 2CL8 was F81. Using Tracer v1.5 we confirmed that burnin occurred at 5 million generations for the MrBayes run and the first 25% of samples were discarded as burnin. Both BI and ML analyses indicated that *L. pyromelana* is composed of two well-supported, geographically distinct clades (Figs. 1 and 2). The more northern clade, here called the Colorado Plateau (CP) lineage, occurs on the Colorado Plateau and adjacent high-elevation areas of northern Arizona, northern New Mexico, Utah and Nevada. In contrast, the southern clade, here called the Sierra Madre Occidental lineage (SMO), is separated from the CP lineage by low desert and is distributed mainly through the Sierra Madre Occidental and isolated sky-island mountains in southern Arizona and New Mexico. Some structure in the SMO clade is noticeable in the mtDNA tree (Fig. 2); however, these subclades are not geographically distinct and not supported using the nuclear genes. Similarly, two smaller clades appear within the CP clade but are not discriminated by the nuclear genes.

All four runs of Structurama demonstrated that most individuals (97%) are correctly classified into one of the two lineages found in the phylogenetic analyses (Fig. 2), although this method predicted three populations based on the highest sampling from the posterior probability (Pp) distribution (1 = 0.0, 2 = 0.29, 3 = 0.52, 4 = 0.16, 5 = 0.02, 6 = 0.00). One individual from Sonora, Mexico (RWB 0867), found in the range of SMO lineage, was classified into a third population. Additionally, an individual from Chihuahua (JLE 14464) from the SMO clade was classified with the CP lineage, consistent with the discordance between nuclear gene tree and the mtDNA/nuclear concatenated tree (Fig. 2). We also ran an admixture model to include the possibility that introgression between recently diverged species is possible. With three fixed populations, results showed that 90.2% of individuals were not admixed and the remaining four admixed samples were assigned to the proper clade, relative to the phylogenetic structure (Fig. 2), at a proportion of 80%. Therefore, according to these markers and sampling, introgression is likely minimal.

The Pp distribution from all runs of BPP using all values for ε, indicated that species were delimited at 100% along the same composition and clades according to the phylogenetic trees (Fig. 2). Burnin occurred by 10,000 generations, and 198,000 samples were used in the posterior probability distribution. In addition, support for the delineation of either clade was zero when samples were randomized by lineage.

3.2. Diversification processes

IMa inferred demographic and temporal patterns based on estimates from 99,000 samples after burnin and attained ESS values for all parameters >70 (most were above 10,000). Estimates were burned in at 100,000 generations. Considering error, our estimates of divergence dates between the two lineages suggest that initial splits likely occurred prior to the major Pleistocene glacial cycles and likely at the end of the Miocene or beginning of the Pliocene, although this prediction must be tempered given the error around the mean date (5.6 Ma, 95% CI = 1.18–13.4 Ma). Additionally, migration in either direction is close to zero (95% CI m_1 = 0.0002, 95% CI = 0.0–0.0006; m_2 = 0.0003, 95% CI = 0.0–0.0010). The predicted effective population size is much smaller in the Colorado Plateau lineage (N_{eCP}; 393,656; 95% CI = 207,708–669,280) compared to the Sierra Madre Occidental lineage (N_{eSMO}; 1,407,157 95% CI: 816,983–2,358,637), and both were smaller than the ancestral population-size estimates (N_{eA}; 1,856,147; 95% CI = 124,624–8,054,446). Likelihood-ratio tests confirmed these differences in population sizes. Using LLR tests of nested models, equal N_e among the extant and ancestral populations were rejected when tested against the full model where all parameters (N_{eSMO}, N_{eCP}, N_{eA}, m_1, and m_2) differed ($P < 0.002$; 2LLR = 13.5–20.8). This agrees with estimates that N_{eCP} is much smaller than N_{eSMO}, and both are smaller than the ancestral population, N_{eA}. Additionally, testing the model where migration is equal between the two lineages against the model where migration is zero for both lineages was not rejected using a mixed χ^2 distribution (2LLR = 1.65, $P = 0.10$).

3.3. Historical demography

To estimate population-size change after speciation, we first determined the appropriate model of evolution for each lineage and each gene using BIC in jModeltest and then approximated the closest model available in Beast 1.5.4 (Drummond and Rambaut, 2007). For the SMO clade, the following models were used: Cytb = HKY + Γ, 2CL8 = HKY + Γ, and PRLR = HKY + Γ. For

Table 1
Basic summary statistics for each gene by lineage (Colorado Plateau = CP; Sierra Madre Occidental = SMO). Since some individuals were missing a significant portion of sequence data (50%), therefore we recalculated summary stats for a subsample of individuals with more complete data (in parentheses). The two values in parentheses after the length of each gene represent (1) the total number of sites used to calculate summary stats prior to reducing the dataset and (2) the number of base pairs used after reducing the dataset for taxa with missing sequence data, respectively.

Gene	Lineage	Length (bp)	Samples*	Polymorphic sites	Haplotypes	π (nucleotide diversity)	Tajima's *D*
Cytb	CP	1117 (945; 1027)	18 (13)	38 (36)	11 (8)	0.010 (0.009)	−0.655 (−0.767; $P > 0.10$)
	SMO	1117 (954; 980)	27 (25)	20 (71)	20 (18)	0.022 (0.021)	0.505 (0.484; $P > 0.10$)
2CL8	CP	505 (391; 397)	18 (17)	6 (6)	5 (5)	0.004 (0.004)	−0.134 (−0.047; $P > 0.10$)
	SMO	505 (212; 391)	41 (34)	3 (7)	10 (5)	0.005 (0.004)	1.54 (0.055; $P > 0.10$)
PLRL	CP	585 (199; 501)	22 (20)	1 (1)	2 (2)	0.0005 (0.0002)	−1.162 (−1.164; $P > 0.10$)
	SMO	585 (235; 430)	41 (36)	7 (7)	9 (9)	0.008 (0.004)	0.349 (0.259 , $P > 0.10$)

* The following samples removed (parentheses) for the genes: Cyt b-FTB 1517, 2082, 2215, 2221, 2249, BYU 49395, T154; PRLR-FTB 2218, 2222 (2 haplotypes), 2223 (2 haplotypes), 2247, BTH 207; 2CL8-MVZ 229713, 229714, FTB 2077, 2081, 2216 (2 haplotypes), 2247, T151.

the CP lineage, the following models were preferred: Cytb = HKY + Γ, 2CL8 = HKY, PRLR = HKY. Population changes through time were estimated with EBSP. To obtain ESS values >200 for all parameters required 40×10^6 generations for the CP lineage and 120×10^6 million generations for the SMO lineage, with a burnin of 10×10^6 generations for both. Estimates of population size through time using EBSP also indicate that population sizes were different among lineages, with the N_e of the CP lineage generally falling under $1x10^6$ (Fig. 3). Additionally, population dynamics for the CP lineage suggest that the population did not change through the Pleistocene. The parameter demographic.populationSizeChanges indicates that zero population-size change was sampled most frequently (0 = 14,500 samples; 1 = 14,200 samples) for this population. In contrast, the single population size change was most often sampled (0 = 25,000 samples; 1 = 55,000) for SMO with a median increase in population size from 2×10^6 to 5×10^6, occurring mostly in the Late Pleistocene. In comparison, estimates from Tajima's *D* were not significant for any gene (Table 1). This indicates that the signature of population expansion or selection for either lineage was not present on these loci. Finally, although the absolute values are likely not comparable between programs, the ratio of early estimates of population size from Beast are similar to IMa, where the CP lineage (N_e = 1728108) is only 34% (28% in IMa) of the size of the SMO lineage (N_e = 5058,299).

3.4. Ecological-niche modeling

Using Maxent, ecological-niche models for both lineages produced test AUC values > 0.7 for all replicates; CP: mean = 0.965, range = 0.940–0.987; SMO mean = 0.990, range = 0.973–0.996. Binomial omission tests were significant for all models for all thresholds ($P < 0.001$). The top predictors for the CP clade were isothermality (22%), precipitation of the coldest quarter (12.6%), mean diurnal range (12.3%) and annual mean temperature (11.1%). Variables with the greatest contributions for the SMO lineage were precipitation seasonality (31.4%), isothermality (20.7%), and precipitation of warmest quarter (15.8%). Predicted ranges of both lineages overlapped substantially (see S1). We calculated predicted ranges and their area of overlap by multiplying the number of pixels representing suitable habitat by 0.86 (30 s of arc equals 0.93 km, thus a 30-s pixel equals 0.86 km^2). Predictions for the range of the CP lineage are 2754,361 km^2, SMO at 2192,125 km^2, and their overlap at 233,294 km^2. The predicted distributions of the CP and SMO overlap by 8.5% and 10.6% of their respective distributions.

We also determined whether low desert habitats exist outside the predicted niche for either lineage. The first three PCA variables produced Eigen values >1, with PCA 1 and 2, occupying 41.69% and 28.65% of variance, respectively. High positive factor loadings for PCA 1 greater than 0.75 for bioclim 1 (Annual Mean Temperature) and 5 (Max Temperature of Warmest Month), and high negative loadings for bioclim 14 (Precipitation of Driest Month) and 17 (Precipitation of Driest Quarter) indicate that this axis separates individuals along a gradient from dry/warm to cool/wet conditions. Alternatively, PCA 2 with high negative loadings for bioclim 4 (Temperature Seasonality) and high positive values for bioclim 18 (Precipitation of Warmest Quarter) suggests a separation of groups along a gradient from high seasonality with lower summer precipitation to low seasonality with higher summer precipitation. The first principal component was similar for the two lineages and was significantly different for the desert samples and the SMO clade (Kruskal-Wallis $H = 65.690$; $P = 0.000$), while the second axis was significant for all groups (Kruskal-Wallis $H = 35.63$; $P < 0.01$; Fig. 4).

For the niche identity test, values of both Schoener's *D* and *I* for our empirical data were significantly lower than the randomized distribution of 100 pseudoreplicates; $P < 2.2 \times 10^{-16}$ for both statistics. This rejects the null hypotheses for niche identity between the CP and SMO lineages. For the background test we conclude the reverse, where empirical values for *D* and *I* were both significantly greater than the distribution of values obtained comparing empirical localities for CP lineage with background estimates for the SMO

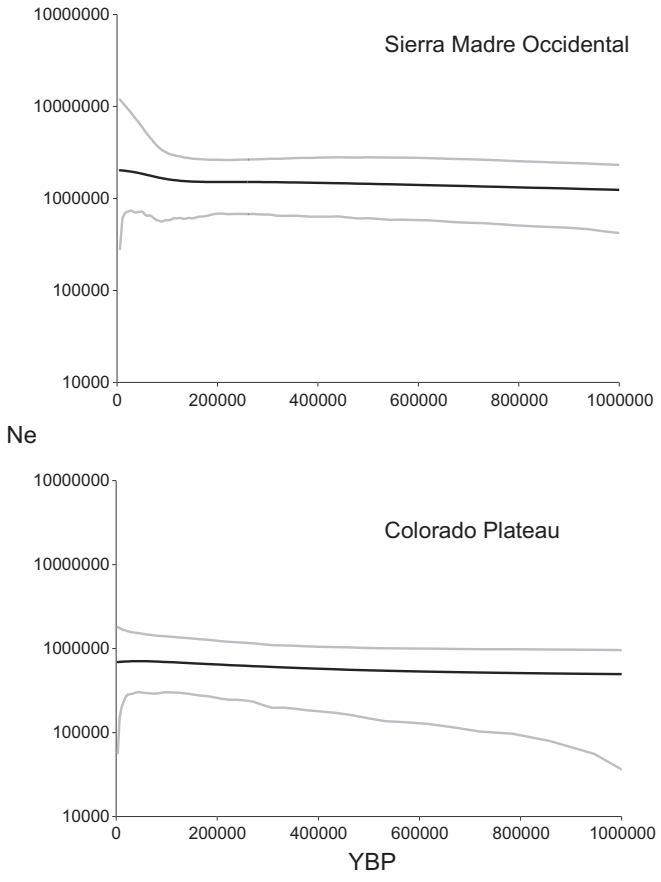

Fig. 3. Extended Bayesian Skyline Plots for the Sierra Madre Occidental (SMO) and Colorado Plateau (CP) lineages showing median and 95% HPD changes in the effective population size (N_e) over time, indicated as years before present (YBP).

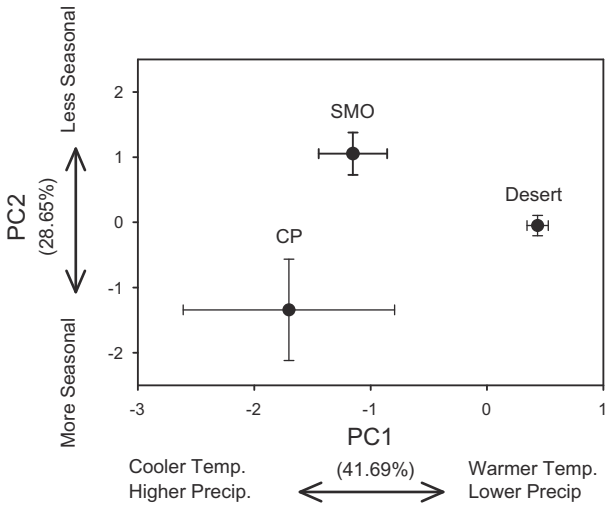

Fig. 4. Results from the PCA showing the means and 95% CIs of scores for samples of the Colorado Plateau (CP) and Sierra Madre Occidental (SMO) clades, as well 100 random points taken from elevations below 1400 m (Desert).

lineage ($D = 0.078$, $P < 4.21 \times 10^{-15}$; $I = 0.241$, $P < 2.2 \times 10^{-16}$). Similarly, the empirical values of D and I for the SMO lineage were significantly larger than the background estimates for the CP ($D = 0.078$; $P < 2.2 \times 10^{-16}$; $I = 0.241$, $P < 2.2 \times 10^{-16}$). These tests indicate that the two lineages do not have identical niches but are likely more similar than random background points drawn from the overall range of each lineage.

4. Discussion

Delimiting species and understanding how speciation occurs are generally decoupled in most studies. Here we show that understanding modes of speciation and delimiting taxa using coalescent based methods compliment one other. Moreover, demographic changes after speciation were examined to clarify potential differences between population demographic trajectories in the separate species. Our results show that the Arizona Mountain Kingsnake is composed of two distinct lineages, one occupying the high areas of the Colorado Plateau (CP) and the other occupying the Madrean sky islands and Sierra Madre Occidental (SMO) to the south (Figs. 1 and 2). The border between these lineages likely occurs below the Mogollon Rim (the southern edge of the Colorado Plateau) separated by low desert from the populations occurring at high elevation to the south (Fig. 4).

Tests using Bayesian coalescent methods to identify cryptic diversity overwhelmingly indicate that *L. pyromelana* is composed of two distinct species. Most phylogeographic-based taxonomic studies rely on the Evolutionary Species Concept (criterion) grounded in the logic of the General Lineage Species Concept (de Queiroz, 2007) to delimit species. The Bayesian Phylogenetic and Phylogeographic (BPP) method provides an adequate test of these concepts, despite the potential impact of gene flow among lineages. Functionally, the limitation of BPP indicates that if gene flow between lineages were extensive, then identification and delimitation of species might not be possible (Yang and Rannala, 2010). Our null tests, by randomizing the lineage identity of individuals, support these conclusions and suggest that organizing *L. pyromelana* into two species is not an artifact of the method. It is unclear how much migration over time between species will cause coalescent methods, which account only for lineage sorting, to fail to detect unique species. Given that these lineages can be delimited likely indicates that the presence of gene flow and hybrid individuals among species is low.

In agreement with the results using BPP, our tests using IMa indicate that little if any gene flow occurred after speciation, either since the origin of the lineages or after potential secondary contact. Diversification at the Mogollon Rim has been examined in numerous taxa (Lomolino et al., 1989; Lamb et al., 1997; Goldberg et al., 2004; Barrowclough et al., 2006; Haenel, 2007; Stevens and Polhemus, 2008), although mode of speciation is often not tested. Given little or no gene flow between lineages of *L. pyromelana* and that neither occur in the low desert, which appears to be outside of their basic niche (Fig. 4), we suggest that isolation at high elevations yielded two species (Fig. 2). Population sizes among ancestral and extant lineages were significantly different, with the estimate of the CP species averaging less than 34% of the size of the SMO species and less than 22% of the ancestral size.

Although changes in climate during the Pleistocene have been invoked for the diversification of many lineages in the Southwest (see Hafner and Riddle, 2008), other studies have found timing to predate the Pleistocene (Bryson et al., 2010; Downie, 2004; Smith and Farrell, 2005). Using coalescent inferences of diversification time, which should account for overestimation of gene divergences (Edwards and Beerli, 2000), we show that speciation in this group might have occurred well before the Pleistocene, with the lower 95% CI (~1.18 Ma) barely encompassing the Pleistocene. Although precision in determining the exact time of diversification is difficult, the mean estimate of speciation in *L. pyromelana* (~ 5Ma) is coincidental with the uplift of the Colorado Plateau and the aridification of the American Southwest (Hafner and Riddle, 2008; Sahagian et al., 2002). Diversification under these arid conditions in this snake is thus supported by two lines of evidence: (1) the timing of speciation and (2) the absence of populations in low desert, xeric habitats (Fig. 4). Like most diversification events in the tribe Lampropeltini (Pyron and Burbrink, 2009a,b), speciation in the Arizona King Snake also predates the Pleistocene. Speciation due to Pleistocene climatic events was once considered the standard mode for faunal diversification in North America; this study adds yet another organism to the list of species that likely diversified prior to the Pleistocene (Klicka and Zink, 1999; McCormack et al., 2010; Pyron and Burbrink, 2009b; Zink and Slowinski, 1995).

We also examined impacts of Pleistocene climate change on population sizes in both lineages. Population contraction during glacial maxima and expansion during glacial minima has been suggested for many organisms in the eastern US (Hewitt, 1996, 2000). However, for high-elevation species in the SW, the pattern should be the opposite, where cooler mesic-adapted species expand their ranges and population sizes during glacial maxima, which subsequently increased cooler and wetter conditions in low-elevation areas (Hewitt, 1996, 2000; Comes and Kadereit, 1998; Betancourt, 1990; Ditto and Frey, 2007). Although the two species of Arizona Mountain Kingsnake appear to occupy similar niches, population size changes differed throughout the Pleistocene. After speciation, demographic dynamics indicate that the CP lineage remained smaller and more stable than the SMO lineage throughout the Pleistocene, whereas the SMO lineage experienced at least one modest change in population size during the end of the Pleistocene (Fig. 3). Therefore, we conclude that unlike those of many other vertebrates (Burbrink et al., 2008; Castoe et al., 2007; Guiher and Burbrink, 2008; Hewitt, 2000, 1996) these population sizes were not negatively affected by major climate changes. This agrees with other research on high-elevation vertebrates ranging outside the direct area of inundation by North American glaciers, where population sizes were not negatively impacted by Pleistocene climate change (Shepard and Burbrink, 2008, 2009).

Understanding processes of speciation helps to strengthen arguments for species delimitation, particularly in cases where gene flow is low and the timing of divergence is ancient (Hey, 2009). Similar to covicariance studies (Hickerson et al., 2006) where pulses of diversification are examined using coalescent methods across a wide variety of taxa in a single area, we envision an extension of this idea, where modes of speciation are enumerated across organisms in particular regions. This would allow us not only to quantify diversity in the region, but also to determine whether single or multiple modes of speciation are common to a barrier given the biology of the organisms as well. For example, diversification across a community at a barrier could occur at single or multiple times but also, given the biology of the species, could happen with limited (parapatry) or no gene flow (allopatry). At least for now, we show that investigating all three patterns (e.g., species delimitation, speciation process, and post-speciation demographic history) is possible in a single study.

Finally, our results indicate that two species occur within the Arizona Mountain Kingsnake, suggesting that taxonomic revision is necessary. The oldest subspecies occurring within the range of the CP lineage is *Lampropeltis pyromelana pyromelana* with the type specimen restricted to Fort Whipple, AZ (near Prescott; Tanner, 1953). Therefore, we suggest that the CP lineage be designated *L. pyromelana*. The remaining subspecies in that lineage, *L. p. infralabialis*, found in northern Arizona, Utah and Nevada should be synonymized with the nominate species (Tanner, 1953). The range of the

SMO lineage overlaps the subspecies, *L. p. knoblochi*, which was initially recognized as a distinct species (Taylor, 1950) and has recently been elevated to species status (Lemos-Espinal et al., 2003). The type specimen for this subspecies is from Mojarachic, Chihuahua, Mexico. Our sample from the type locality is within the SMO clade (Fig. 2) and falls within the range of that clade. We therefore suggest that *Lampropeltis knoblochi* be recognized as a distinct species with the range corresponding to the SMO lineage. Likewise, our samples from the type locality of the remaining younger subspecies, *L. p. woodini* (Carr Canyon, Huachuca Mountains, Conchise Co., AZ; Tanner, 1953), fall within the range of *L. knoblochi* in southeastern Arizona. We suggest that *L. p. woodini* therefore be synonymized with *L. knoblochi*.

5. Conclusions

This research delimits species using coalescent methods, defines processes associated with speciation, and estimates post-speciation population demographics. In the Arizona Mountain Kingsnake, *Lampropeltis pyromelana*, we demonstrate with the BPP method of Yang and Rannala (2010) that two deep lineages found on the Colorado Plateau and the Sierra Madre Occidentals/Madrean sky islands correspond to two distinct species. These species likely diverged prior to the Pleistocene following the uplift of the Colorado Plateau and aridification of the SW. Isolation and migration models suggest that diversification was accompanied by little or no gene flow and that population size in the Colorado Plateau lineage was much smaller than the other lineage. Our results also indicate that these species were isolated by unfavorable conditions in the low desert and likely diverged allopatrically. Additionally, population sizes in both lineages remained stable through the Pleistocene.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ympev.2011.05.009.

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