Ecological divergence across two North American biodiversity hotspots in the Yellow-Bellied Kingsnake (Lampropeltis calligaster)

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Ecological divergence in the yellow-bellied kingsnake (*Lampropeltis calligaster*) at two North American biodiversity hotspots

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**A B S T R A C T**

Several biogeographic barriers in the Eastern Nearctic appear to reduce gene flow among populations of many species in predictable ways, however these patterns used to infer process of divergence may be deceiving if alternative modes of diversification are not considered. By using a multilocus statistical phylogeographic approach to examine diversity within a North American snake, *Lampropeltis calligaster*, we find that mode and timing of speciation near the Mississippi River embayment and peninsular Florida, two main biodiversity hotspots in eastern North America, challenge previously held notions of strict vicariance as the causal factor behind patterns of divergence seen among taxa at these locations. We found three species inhabiting distinct ecological niches with divergences dating to the mid- and early-Pleistocene with subsequently stable or increasing effective population sizes, further supporting the idea that the Pleistocene was an important driver of diversification in North America. Our results lead to a revised hypothesis that ecological divergence has occurred in this group across environments associated with the Mississippi River and at the Florida peninsula. Importantly, in their western distributions, we show that species divergence is associated with the ecological transition from distinct forested habitats to grasslands, rather than the nearby Mississippi River, a barrier often implicated for many other organisms. Additionally, we stress the importance of examining each delimited lineage with respect to conservation, since ecological niche models suggest that by the end of the century changes in climate may negatively alter habitat suitability and, barring adaptation, substantially reduce the suitable range of two of the three species we identified.

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1. Introduction

Geographic barriers to gene flow that isolate populations and generate species have been characterized using phylogeographic analyses (Avise, 2000), which play a strong role in understanding how species form with regard to timing, degree of isolation (allopatric or parapatric) and location, while also identifying previously undescribed or unrecognized diversity (Pyron and Burbrink, 2010). In areas such as the Nearctic, physical and ecological barriers have been examined across many taxa and appear to isolate populations, reducing gene flow across organisms in similar ways (Avise, 2000; Pyron and Burbrink, 2010). For wide-ranging taxa, one or more of these barriers is often found to influence population structure predictably. Causes for these repeated patterns observed among taxa may be deceiving in that they appear to have similar superficial effects but vary in key characteristics such as fine-scale location, timing or mode of divergence. Examining these key characteristics associated with biogeographic barriers enhances our understanding of regional processes related to speciation. Certainly, patterns may appear repeated among taxa, but underlying processes may vary greatly for each individual species. In eastern North America, there are several major areas where terrestrial taxa show strong overlapping patterns of divergence. First, the Mississippi River region is particularly important for isolating populations and generating species (Pyron and Burbrink, 2010; Robison, 1986). During periods of climatic change in the Pleistocene, the volume of glacial meltwater and the river bed itself was subject to fluctuation in size and location (Royall et al., 1991; Smith, 1996). The current prevailing hypothesis is that the
Mississippi River was instrumental in generating biodiversity in eastern North America (Burbrink et al., 2000; Fontanella et al., 2008; Gamble et al., 2008; Guiher and Burbrink, 2008; Howes et al., 2006; Jackson and Austin, 2010; Leaché and Reeder, 2002; Lemmon et al., 2007; Pyron and Burbrink, 2009; Walker and Avise, 1998). Congruently, a longitudinal ecological transition zone from the central prairies to the eastern Nearctic forests suggests the possibility that ecological divergence in these different habitats drove speciation. This transition zone along the upper portions of the river was affected by glacial events during the Pleistocene and may not represent a long term, stable barrier (Robison, 1986; Royall et al., 1991), therefore the processes that isolated populations on separate sides of the Mississippi River (Burbrink et al., 2000; Fontanella et al., 2008; Guiher and Burbrink, 2008; Pyron and Burbrink, 2009; Ruane et al., 2014) may be due to ecological transition and not historical vicariance associated with the river itself. Both processes may be at work and the shared phylogeographic structure observed in codistributed taxa may only be pseudocongruent, indicating that spatial distributions of lineages are broadly similar across the Mississippi River, but may differ in mode, timing or actual location of speciation (Pyron and Burbrink, 2010; Soltis et al., 2006).

Another major region important for isolating populations in the Eastern Nearctic is located where the Florida peninsula meets the continental US (Fontanella et al., 2008; Guiher and Burbrink, 2008). The patterns of divergence there are typically explained by refugial isolation on islands in northern and central peninsular Florida during periods of elevation in sea level during the Oligocene, Pliocene and early Pleistocene (James, 1961; Soltis et al., 2006). Furthermore, this area is home to multiple rivers that may have been responsible for limiting secondary contact (Soltis et al., 2006). Alternatively, the Florida peninsula features an area of fairly sharp ecological turnover where major climate zones and ecoregional transitions occur (Omernick, 1987). This ecological transition from the southern coastal plain to the Piedmont may be a causal factor driving regional ecological speciation, particularly in groups with key characteristics that are not well explained by traditional island or riverine vicariance.

Although there is disagreement about the role of the Pleistocene in generating modern diversity (Klicka and Zink, 1997), recent work has shown that changes in climate during the Pleistocene may have significantly driven diversification, especially within the common snakes of the genus Lampropeltis (James, 1961; Soltis et al., 2006). Estimating timing of diversification while including post-divergence demographies also helps determine how populations have responded to climatic cycles. Testing these hypotheses to assess the importance of the Pliocene and Pleistocene in shaping current biodiversity requires credible estimates of speciation times within taxa to accurately reflect evolutionary history.

To elucidate processes of diversification associated with these barriers at the Mississippi River region and in the Southeastern United States (SEUS), it is important to first examine organisms with distributions that cross these transition zones and respond to landscape features. Snakes are exceptional candidates for studies on the effect of ecology and landscape isolation; they are known to respond both to vicariant barriers and ecological transitions (Brandley et al., 2010; Burbrink et al., 2011). The yellow-bellied kingsnake, Lampropeltis calligaster (Harlan, 1827), is found across much of the southern United States and three subspecies are currently recognized, distinguished by color pattern and average number of infralabial scales (Blanchard, 1921; Price, 1987). This morphological variation also corresponds to areas on either side of ecological transitions associated with the Mississippi River, and near the Florida peninsula, offering an opportunity to test associated patterns and processes of speciation at these two biodiversity hotspots. The eastern subspecies of L. calligaster, the Mole Kingsnake, Lampropeltis c. rhombomaculata (Holbrook, 1836) is generally found east of the Mississippi River and on the Piedmont and eastern seaboard, while Lampropeltis c. calligaster (Harlan, 1827), the Prairie Kingsnake, is primarily found in the eastern Great and Central Plains from Texas, Oklahoma and Kansas east to Indiana (Fig. 1). The two subspecies meet east of the Mississippi River, where morphological evidence suggests ranges overlap and hybridization occurs (Cook, 1945). The eastern and western subspecies were both originally described as unique species (Harlan, 1827; Holbrook, 1836) but were reduced to subspecies by Cook (1945) based on intergradation and parapatric distribution in the eastern portion of the State of Mississippi. A third and much rarer form, Lampropeltis c. occipitolineata (Price, 1987), the South Florida Mole Kingsnake, is poorly represented in museum collections and is found in only a few counties in South Florida (Krysko et al., 2000; Price, 1987). Importantly, the former two taxa were considered distinct species occupying distinctly different niches (Wright and Wright, 1957) prior to being arbitrarily delimited to subspecies (Cook, 1945).

While statistical species delimitation is becoming common (Fujita et al., 2012), and it is clear that even in North America where surveys of species diversity have been conducted for more than 200 years, phylogeographic studies are still demonstrating that a substantial amount of biological diversity remains undiscovered in terms of uncovering numerous cryptic lineages and populations (Burbrink and Guiher, 2015; McKelvy et al., 2016; Myers et al., 2013a; Pyron and Burbrink, 2009; Ruane et al., 2014). However, the potential fate with regard to conservation of newly recognized species is rarely tested. An often-overlooked consequence of uncovering cryptic diversity is that newly erected taxa are subsets of the total range of the species complex from which they were delimited. Distinct scenarios for each lineage regarding the influence of climate change and urbanization are likely. Conservation efforts to facilitate the survival of these species must reflect the best available data (Malaney and Cook, 2013). Recent discoveries of cryptic lineages with confined ranges are not rare, and small range size is understood as a factor correlated with extinction (Biely et al., 2008; Niemiller et al., 2013); if researchers and stakeholders are expected to incorporate newly uncovered taxa into management plans, available information, including niche trajectories modeled from available climate data, should be examined.

In this study, we examine the phylogeographic history of the L. calligaster complex to first determine if distinct lineages occur at known geographical barriers. We then test specific causes of lineage diversification from among several potential candidate hypotheses. To do this, we investigate divergence times to assess if diversification corresponds with major Pleistocene events. We use redundancy analysis and Mantel tests to determine if the pattern of divergence we observe at the Mississippi River region is best explained by a model of vicariance at the river or by divergence across an ecological gradient. To test mode of speciation, we explore the niche identity of each species in a biogeographic context via environmental niche modeling and assess if gene flow is asymmetric between delimitable species in adjacent niches. We then use historical demographic analyses to determine if populations from different species respond to climatic fluctuations in similar ways. We generate an estimate of future suitable climatic space across North America, and discuss conservation implications due to changes in potential range size for the rare occipitolineata form of L. calligaster. Our results lead to a better understanding of what causes diversification at major biogeographic transitions in eastern North America.
2. Materials and methods

2.1. Sampling, sequencing and gene trees

We obtained tissue samples from 60 individuals from across the known distribution of Lampropeltis calligaster (Fig. 1; Supplementary Material 1); no protected or endangered species were involved in the work. Using 4.5 mm tissue subsample from each, we extracted DNA using Qiagen DNeasy kits following the provided protocol for animal tissues. We amplified one mitochondrial gene (Cytochrome b) and four nuclear loci (CLLAT, NT3, PRLR and VIM56) via polymerase chain reaction (PCR) using temperatures and primer sequences shown in Table 1. PCR products were cleaned using 1.5 l of ExoSap-IT (USB Corporation). We used 2 l of primer, 5 l of water and 2 l of Beckman Coulter DTCS template DNA on a Beckman Coulter CEQ 8000 Separation Module, System ID 490164 to sequence. We supplemented these data with sequences obtained in a previous study on Lampropeltis (Ruane et al., 2014) (Supplementary Material 2), and used the program Geneious v4.8.5 (Kearse et al., 2012) with the Geneious alignment algorithm to align all sequences. After conservatively trimming any sequences with missing data, we used PHASE v2.1.1 (Stephens and Donnelly, 2003) to determine the most probable pairs of alleles in the nuclear loci. We transformed files from FASTA alignments to Phase input files using the online converter Seqphase (Flot, 2010) and ran them multiple times to ensure consistency using the default threshold of 90%. We used these data in all genetic analyses. We estimated individual gene trees in BEAST v. 1.7.5 (Drummond et al., 2012). We determined best fit models of nucleotide substitution for each locus using ranked BIC scores in jModeltest 2.1.4 (Darriba et al., 2012).

2.2. Species delimitation and divergence dating

To first find the number of distinct populations, we assigned individuals to genetic clusters without constraining them to spe-
cies using Strucrurama (Huelsenbeck et al., 2011). We ran Strucrurama five times on the phased multilocus dataset using 10,000,000 MCMC cycles and three chains with prior populations k = 1–4, as well as five times without mtDNA to ensure that support values did not differ significantly, that the results were not driven exclusively by mtDNA and to reduce the chances that upstream processes influence delimitation (Olave et al., 2014). We sampled every 10,000 generations with a burn-in of one million generations removed.

We estimated divergence time and phylogeny in BEAST using the groups assigned in Strucrurama and implemented in the program BEAST v. 1.7.5 (Drummond et al., 2012), ultimately to be used as a guide tree to delimit taxa (see below). All loci were included and we used the outgroups Cemophora coccinea and Lampropeltis extenuata based on Ruane et al. (2014). We estimated tree shape under a Yule prior, population size under a piecewise linear model with constant root, and assigned each locus the appropriate model estimated by jModelTest (i.e., HKY or HKY + Γ). We placed a fossil calibration at the node uniting Lampropeltis from Cemophora as described in detail in Myers et al. (2013a). We estimated species trees in a series of 10 concurrent runs of 200 million generations each and combined with LogCombiner (Drummond et al., 2012), totaling two billion generations sampled every 200,000 generations. Burn in at 20% was removed with LogCombiner (Drummond et al., 2012) and TreeAnnotator (Drummond et al., 2012), and when assessed in Tracer v1.5 (Rambaut and Drummond, 2007), effective samples size (ESS) scores were 1000 or higher for all parameters. Additionally, we generated admixture proportions for eastern and western lineages (k = 2) in the program Structure (Pritchard et al., 2000) and used phylo.to.map in the R package phytools (Revell, 2012) to integrate and present admixture information with the phylogenetic tree across the range of the species.

To delimit species groups inferred by Strucrurama, we used BP&P (Yang and Rannala, 2010), implementing an RJMCMC model to estimate the posterior probability of nodes using the species tree we generated in BEAST. We used the species delimitation algorithm and allowed the fine-tuning parameters to automatically adjust so that swapping rates were between 0.30 and 0.70. We used three combinations of priors for ancestral population size and root age: large ancestral population with recent divergence (1,10)(2,2000), large ancestral population with ancient divergence (1,10)(1,10), and small population size with recent divergence (2,2000)(2,2000). We ran both nuclear and nuclear + mitochondrial datasets five times to ensure that support values did not differ significantly and randomized lineage designations to ensure node support was not based solely on priors. We also ran BP&P without our guide tree to provide a posterior probability for number of species (Yang and Rannala, 2014).

To verify results across species delimitation methods, we also used the program STACEY (Jones, 2016) implemented in BEAST 2.4.2 (Bouckaert et al., 2014). STACEY improves the efficiency of the DISSECT model (Jones et al., 2015) to delimit species and infer species trees without guide trees. For the STACEY run, we used the HKY model of evolution, all ploidy scalars at 2.0 for nuclear genes, and HKY tree model with collapse height of 0.0001, birth diff rate of 100.0, relative death rate and collapse weight at 0.5 and origin height of 100.0. We assigned lognormal priors to growth and clock rates instead of 1/x as described in the tutorial and a relative death-rate prior as uniform between −0.5 and 0.5. We ran the MCMC chain for 100 million generations, storing the state to disk every 10 million generations and logging trees every 50,000 generations.

Additionally, we used the genealogical sorting index (GSI) test (Cummings et al., 2008) to measure degree of exclusive ancestry of each delimited taxon on a scale of 0 (non-exclusive) to 1 (monophyletic) to determine how often each gene tree agrees with coalescent species delimitation.

### 2.3. Historical demography and migration

Most diversification within the genus Lampropeltis occurred in the Pleistocene (Ruane et al., 2014), with subsequent population size stability or expansion (Burbrink et al., 2011; Myers et al., 2013a). To understand historical demography and confirm that this is also true for L. calligaster, we investigated changes in effective population size over time (Nₑ) in delimited taxa using Extended Bayesian Skyline Plots (EBSP) in the program BEAST v.1.7.5 (Drummond et al., 2012). Each locus was modeled using results from jModeltest and the parameters specified in the tutorial for EBSP. We scaled time by using an estimated mitochondrial substitution rate of 1 × 10⁻⁸ per site per generation (Eo and DeWoody, 2010). The dataset was run for 500 million generations, and all ESS values analyzed in Tracer v1.5 (Rambaut and Drummond, 2007) were greater than 200.

To test gene flow between lineages occurring in distinctly different niches, we determined which direction migration may be occurring between adjacent eastern and western lineages by employing the program Migrate 3.6 (Beerli and Palczewski, 2010). Migrate tests four models of migration: panmictic population, bilateral migration and two models of asymmetric migration (Beerli and Palczewski, 2010). We set inheritance scalars for mitochondrial and nuclear genes to reflect that our mitochondrial gene has 1/4 the effective population size of our nuclear genes. Four heated chains were used for MCMC analysis, with values of 1, 3, 10 and 100,000. We summarized results over 10 runs and compared them to ensure stationarity, and checked resulting output files to ensure posterior distributions and marginal likelihood scores indicated burn-in was achieved.

Additionally, to better understand migration given isolation with ancestry, we explored 24 nested parameter models (Supplementary Material 3) of migration and divergence in the program IMa2p (Sethuraman and Hey, 2016) and calculated AIC scores to determine which parameter estimates were most likely given the molecular data. We provided a scaled mutation rate estimate on the mitochondrial gene of 1 × 10⁻⁸ set inheritance scalars of 0.25 for the mitochondrial gene and 1 for nuclear genes, assigned the HKY models of molecular evolution from jModeltest, a generation time of three years (Ernst and Ernst, 2003) and ran the program in M mode 5 times with a random seed, using four CPU processors.
cores and ten chains per core, assigning the recommended heating parameters for medium-sized datasets. We used log-likelihood ratio tests on our 200,000 generated genealogies to determine which of the 24 nested models were best supported and repeated this process four times to ensure consistency.

2.4. Ecological niche modeling

To model the niche of *L. calligaster* and test ecological associations for each lineage, we used all known locations of *L. calligaster* collected from records available through the HerpNet data portal with 35 bioclimate variables from the CliMond Database (Kriticos et al., 2012) to create ecological niche models (ENMs). We assessed correlation of variables using ENMTOOLS (Warren et al., 2010), and all strongly (>0.89) correlated variables were removed, leaving 15 bioclimate variables on which to base our models. Individuals were assigned to populations by range, consistent with subspecies designation. We downsampled potentially erroneous records and duplicates, as well as points in the same cell from the dataset, leaving 155 georeferenced localities for the eastern group, 340 localities for the western group and 17 localities for the South Florida lineage.

We then used the R package spThin (Aiello-Lammens et al., 2015) to thin our dataset spatially to remove sampling bias yet retain as many records and variation as possible. To recover evenly-spaced datasets for our eastern and western groups, we used a 30k threshold in the algorithm, and used a 10k threshold for our rare South Florida localities. For all groups, we repeated our dataset generation 100 times and evaluated them using the function plotThin to ensure that the number of records had plateaued. This left us with 82 records for the western group, 39 records for the eastern group, and 8 for the South Florida lineage (Supplementary Material 3). We trimmed our area of extent to include only the regions where *Lampropeltis calligaster* is distributed in eastern North America, created datasets of 10,000 pseudoabsences around our datasets using the R command BIOMOD_FormatingData in the package Biomod2 (Thuiller et al., 2013), and used Maxent v3.3.3 (Phillips and Dudik, 2008) to construct initial ENMs as it has been shown to be robust in creating species distribution models from presence only data and from small datasets (Hernandez et al., 2006; Wisz et al., 2008). We replicated our runs 500 times for each group, reserved 30% of samples as a training dataset, created response curves and jackknifed our data to measure variable importance. To ensure that our results were robust, we then used an ensemble modeling approach with the function BIOMOD_EnsembleForecasting from the package Biomod2 (Thuiller et al., 2013), which combines a generalized linear model, generalized additive model, generalized boosting model, classification tree analysis, artificial neural network, surface range envelope, flexible discriminant analysis, multiple adaptive regression splines, maximum entropy and a random forest model into a single output to create the final projections we used to evaluate species distributions. We used 0.7 as our ROC score quality threshold evaluation metric.

We then used ENMTOOLS (Warren et al., 2010) to test whether lineages occupy identical or distinct environmental niches. The niche identity test examines whether niches drawn randomly from the combined samples are significantly different from those drawn from the dataset. The range break test draws random lines through a pooled sample range and estimates degree of similarity to test if populations are linked to either side of a sharp environmental transition (Glor and Warren, 2011). Relative rank as well as summary statistics I and Schoener’s D were generated for each repetition and the observed overlap in niche was compared to this distribution (Glor and Warren, 2011). If observed overlap values fell outside of the 95% confidence interval of the null distribution, the null hypothesis was rejected.

To estimate how the environment and the predicted niche of these organisms may change through time as a result of global climate change, we projected our ensemble models onto bioclimate data from the estimated IPCC fourth assessment report models of climate change for the year 2100 (Susan, 2007). We estimated change in size of suitable habitat by quantifying change in number of 30 s cells from the ENM outputs with extracted score 0.4 or higher (the value at which the known range of the species is best represented) in both the current and future niche predictions, as well as the intersection between the two, by summing the counts of the floating point rasters using the Int Spatial Analyst Tool in ArcGIS. Clamping mask estimates revealed few variables outside their training ranges were unlikely to have a large effect on predicted suitability scores.

To determine which fine scale habitat types are associated with the presence of *L. calligaster* for each detected lineage, we used ArcGIS (ESRI, 2011) and all locality records to extract specific habitat information from GAP land-cover data, a dataset provided by the USGS that focuses on habitat identification (Homer et al., 2004). GAP land-cover data are likely highly dependent on the climate variables recorded in the WorldClim database, but differ from that of strict environmental climate data in that they identify objectively defined ecosystem types based on remote sensing and survey data of flora. This information provided names of the ecosystems and fine-scale habitats in which specimens are found. Habitat results were aggregated by type and tallied. Localities falling on currently developed or otherwise impermeable surfaces were discarded.

2.5. Ecological speciation versus riverine vicariance

To determine if patterns of divergence at the Mississippi River Embayment are best explained by ecological divergence or vicariance associated with the Mississippi River itself, we performed both a traditional Mantel test and a redundancy (RDA) analysis, which allowed us to test how much of our variance is explained by each of the competing hypotheses using matrices of environmental, geographic and genetic data (Legendre and Fortin, 2010). We extracted environmental and geographic data for each individual from the layer files used for niche modeling. We performed a principle component analysis (PCA) on the extracted bioclimate data and used the distance of the first axis (92% of the variance), in our environmental matrices. After importing our datasets into R (Team, 2000), we created distance matrices using the dist function in the R package ade4 (Thioulouze et al., 1997). These tests determine correlation between variables; significant correlation in a Mantel test (p < 0.05) and large values for constrained results in RDA indicate strong relationships between matrices. Results showing values with high correlation between spatial distribution and genetic distance matrices would indicate divergence resulting from riverine vicariance as the primary driver of speciation (or isolation by distance), while correlation between environmental niche and genetic distance matrices would support ecological speciation. We used the functions Mantel.rtest in the package ade4 with 9999 replicates and function rda in the package vegan (Oksanen et al., 2007) to test which matrices (ecological distance or spatial distribution) best describe the variation in the data. Using the same variables we also examined the influence of ecology, geographic distance and vicariance using both standard and partial RDA to isolate the effects of variables and tested significance with the anova function.

3. Results

3.1. Sampling, sequencing and gene trees

We sequenced 2694 base pairs (bp) of DNA from five unlinked loci and estimated the best-fit models of substitution and gene
trees for each locus (Table 1). Of 60 individuals, one or more loci could not be recovered from 29 samples (Supplementary Material 1), resulting in 12% missing data. The gene CLAT was difficult to amplify across all individuals and therefore this gene comprised 58% of missing data, but was retained for all subsequent analyses.

3.2. Species delimitation and divergence dating

All runs of Structurama for both mtDNA and nDNA revealed that most individuals (98%) are classified into western, eastern and South Florida lineages (Fig. 1). Support for three populations was highest at 65% posterior probability, then four populations at most likely (Table 3). A model of east to west and west to east were that equal migration between eastern and western populations is supported by similar likelihood scores, and taking the product of M

58% of missing data, but was retained for all subsequent analyses.

3.4. Ecological niche modeling

The identity test showed observed values for I and D were significantly different from the null distribution (t-test, p < 0.05), indicating that eastern and western lineages as well as eastern and southern lineages occupy distinct ecological niches in North America. The observed values for the range break test also fell outside the 95% distribution of the null, indicating that there is a transition in ecological niche between eastern and western lineages. The variables that contributed most to the projections (Supplementary Material 7) for Lampropeltis calligaster were: highest weekly radiation, mean temperature of warmest quarter and isothermality. For L. rhombomaculata: lowest weekly moisture index, mean temperature of driest quarter and highest weekly radiation. For L. occipitolineata: precipitation of warmest quarter, annual mean temperature and highest weekly radiation.

Tests for fine scale habitat occupancy using GAP data revealed (Fig. 3) that western L. calligaster were directly sampled primarily in prairie, pasture and shrublands (61.4%, n = 86) and secondarily (37.9%, n = 53) in mesic forests. Lampropeltis rhombomaculata was sampled primarily in temperate oak and pine forests (61.4%, n = 41), and secondarily in pasture (32.8%, n = 21). Lampropeltis occipitolineata was sampled primarily in freshwater wet meadow (37.5%, n = 3) and pasture (37.5%, n = 3) and secondarily in southeastern warm temperate forest (25%, n = 2).

Future climate models predicted that core habitat for L. calligaster will remain stable over 99.0% of its range, with overall suitable climatic space increasing 69.1% in distribution northward and eastward across the range currently occupied by L. rhombomaculata (Fig. 4). Climate models predict that suitable niche for L. rhombomaculata and L. occipitolineata will be reduced drastically, as no cells in future climate models for L. occipitolineata contained suitability scores higher than 0.4 (100% reduction) and only a few cells contained suitable scores for L. rhombomaculata (98.5% reduction). Core habitat will also be reduced, with only 7.0% of current niche space remaining suitable under projected conditions. Number of cells are reported in Table 4.

3.5. Ecological speciation versus riverine vicariance

The results from both the Mantel test and the RDA suggested that the variation seen between L. calligaster and L. rhombomaculata is best described by divergence in environment rather than riverine vicariance or isolation by distance. Mantel tests indicated that only ecological distance and genetic distance matrices were significant (p = 0.04, geographic distance p = 0.49). Our RDA analysis also returned similar results, with ecological and genetic distance explaining 86.7% of the data (p = 0.054). Results for the influence of the Mississippi River show that riverine divergence (position east or west of the Mississippi River) only explained 2.8% of the results (p = 0.886), indicating that the current position of the river itself likely did not play a strong role in isolating these species (Supplementary Material 8).

4. Discussion

4.1. Historical biogeography and phylogeography

Speciation in the Eastern Nearctic is complex and processes generating observed patterns may not be obvious. Our results provide a better understanding of the historical role of the biogeographic barriers near the Mississippi River and at the Florida
peninsula with respect to generating ecologically distinct species. Strict vicariance may not be sufficient to describe processes generating distinct lineages for codistributed taxa in regions where habitat transitions are tied closely with a clear isolating barrier. Large barriers such as the Mississippi River are frequently cited as driving regional divergence, but specific tests should be used to account for pseudocongruent effects of underlying processes (Soltis et al., 2006). Results for the _L. calligaster_ complex suggest that ecological diversification, rather than the prevalent hypothesis

![Admixture map and phylogeographic relationships among taxa](image)

**Fig. 2.** Admixture map and phylogeographic relationships among taxa. Individual gene trees are reported in Supplementary Material 1.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Genealogical Sorting Index (GSI) and supporting p-values (in parentheses) for detected lineages. Individual gene trees were compared to the species tree.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>East</td>
</tr>
<tr>
<td>Cyrb</td>
<td>1 (0.0001)</td>
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<tr>
<td>CLLAT</td>
<td>0.8056 (0.0001)</td>
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<tr>
<td>NT3</td>
<td>0.6378 (0.0001)</td>
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<td>PRLR</td>
<td>0.4443 (0.0001)</td>
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<tr>
<td>VIM56</td>
<td>0.2495 (0.0001)</td>
</tr>
</tbody>
</table>

| Table 3 | Model selection results from the program Migrate. Bezier approximation scores, natural log Bayes factors (LBF), model probability and the rank of each model are reported. A model of equal migration between both populations had the most support. |
|---|---|---|---|
| Migration model | Bezier | LBF | Model probability |
| Equal | -4924.32 | 0 | 1.000000 |
| West to east | -4960.04 | -35.72 | 0.000000 |
| East to west | -4960.21 | -35.89 | 0.000000 |
| Panmixia | -5145.41 | -221.09 | 0.000000 |

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A.D. McKelvy, F.T. Burbrink/Molecular Phylogenetics and Evolution 106 (2017) 61–72
of riverine vicariance, drove speciation. Therefore it is possible, particularly in the Eastern Nearctic, that many regionally co-distributed species with similar distributional patterns of lineages may have been generated from potentially different processes giving rise to pseudocongruent patterns (Pyron and Burbrink, 2010).

Here, we determined that speciation has occurred at two major biodiversity hotspots in North America in Lampropeltis calligaster, with each of these lineages currently occupying distinct niches. Coalescent delimitation as well as ecological and morphological (Blanchard, 1921; Price, 1987) data indicate that Lampropeltis calligaster is composed of three closely related species, historically considered distinct taxa (Blanchard, 1921; Holbrook, 1836; Schmidt and Davis, 1941): L. calligaster in the western prairies, L. rhombomaculata in the eastern forests, and L. occipitolineata in South Florida wet prairies.

While it is possible that the Mississippi River was the initial cause of speciation between L. calligaster and L. rhombomaculata, ENMs indicate that each species currently occupies a distinct ecological niche in Eastern North America. GAP land-cover data indicates that prairies and pastureland are preferred habitats for L. calligaster in the west, and dry oak and pine forests are preferred for L. rhombomaculata in the east. Signatures of migration suggest that speciation in these taxa was either accompanied by gene flow...
where niches overlap or has resumed in secondary contact. This separation in niche coupled with climatic shifts may have driven speciation. Redundancy analyses revealed that genetic divergence is best described by environmental factors and only poorly by riverine vicariance. Interestingly, other species pairs reveal divergence near the Mississippi River but diversification was likely driven by habitat changes; Burbrink and Guiner (2015) found a transition in lineages of copperhead snakes (Agkistrodon contortrix) found east of the Mississippi River that roughly corresponds to the same area of divergence estimated in this study between Lampropeltis calligaster and L. rhombomaculata. Divergence times between copperhead lineages were reported overlapping those of L. calligaster at 0.96 MYA (0.46–1.56 MYA), indicating that both L. calligaster and Agkistrodon contortrix lineages may have diverged at a similar time and area during the Pleistocene, suggesting congruent patterns of diversification. It is also therefore likely that the Mississippi River itself may not have been the driver of speciation, as indicated in other taxa (Burbrink and Guiner, 2015; Burbrink et al., 2000; Guiner and Burbrink, 2008), but rather the effect of ecological divergence into unique habitats found on each side of the river. Studies revealing vicariance at or near this barrier will benefit from including alternative models of ecological speciation in hypothesis testing.

We demonstrate roughly equal rates of historical migration between eastern and western species. This information corroborates Cook (1945), who found morphological evidence that a small contact zone composed of intermediate phenotypes (considered hybrids) between L. calligaster and L. rhombomaculata exists east of the Mississippi River. The frequency of this migration, ~0.39 individuals/generation (Migrate) and 0.4–0.9 (lm2a), corresponds to that of other similarly distributed species in the genus Lampropeltis (Ruane et al., 2014) and is lower than the threshold of one to ten individuals per generation required for panmixia (Mills and Allendorf, 1996). Existence of gene flow is corroborated by our admixture results, (Fig. 2), with admixed individuals present on the leading edge of the contact zone of these two species. Low levels of migration may slow rates of ecological speciation and be detrimental if species accumulate non-adaptive alleles in their respective ecoregions, or beneficial, in that migration allows introduced alleles to be acted upon by selection (Schluter, 2001).

The oldest divergence, that of L. calligaster, corresponds with the Pleistocene or late Pliocene at 1.55 MYA (95% HPD = 0.635–2.921 MYA) and corroborates the hypothesis that the species diversity currently found within the genus Lampropeltis calligaster and L. rhombomaculata was likely generated during the Pleistocene (Ruane et al., 2014). This finding adds further evidence that Pleistocene climatic cycles may have been instrumental in generating species diversity, a controversial sub-hypothesis in hypothesis testing.

4.2. Conservation implications

Urbanization of the Florida landscape since 1960 has been faster than anywhere else in the US, with human population doubling every 20 years (Reynolds, 2001). Many species are threatened due to urbanization, and coupling this process with climate change imperils many additional species in Florida (Reece et al., 2013). While current and future niche projections of L. calligaster indicate stable core habitat and increasing overall range, estimates of future climate reveal a troubling prediction for Lampropeltis rhombomaculata and Lampropeltis occipitolineata: areas once found suitable may change drastically over the next century. Assuming no adaptation, climate models indicate that there will be minimal preferred niche space remaining for these two species in 2100. Taxa such as L. occipitolineata locked in a rapidly developing peninsula may be unable to migrate or adapt quickly enough to handle the effects of climate change and urbanization (Parmesan, 2006). Niche evolution is likely far outpaced by climate change (Quintana and Wiens, 2013), uncovering the potential range shifts in these species highlights the downstream implications of species delimitation.

4.3. Taxonomic conclusions

Species delimitation reveals that L. calligaster is composed of three taxa that correspond with previously described subspecies. These three lineages also occupy unique niches in North America and display quantifiable differences in morphology (Blanchard, 1921; Price, 1987). We thus elevate Lampropeltis calligaster, Lampropeltis rhombomaculata and Lampropeltis occipitolineata as species.

Low sample sizes have been shown to be sometimes problematic when dealing with species delimitation (Rittmeyer and Austin, 2012). Due to sampling here, recognition of Lampropeltis occipitolineata may be considered by some as premature, however, in addition to these coalescent methods identifying and supporting Lampropeltis occipitolineata as a distinct species, the species shows a 500 km separation in range from L. rhombomaculata and exists in a distinctly different niche, displaying striking morphological differences from its sister lineage (Price, 1987). We therefore recommend that this taxon be elevated to the species level.

4.4. Species synonymy and distribution

4.4.1. Lampropeltis calligaster (Harlan, 1827)

Lampropeltis calligaster (Harlan, 1827), Prairie Kingsnake. Type locality: “Missouri”, restricted to “vicinity of St. Louis” by Schmidt (1953). No holotype designated. Three syntypes (Ophibolus evansi) have been designated (Blaney, 1979); USNM 1593, an adult male, an adult and a juvenile female, collected in central Illinois by R. Kennicott, in 1855.

Coluber calligaster Harlan, 1827:359.


Distribution: Lampropeltis calligaster is composed of all populations west of the Mississippi River in the plains states and crosses the Mississippi River embayment east to Illinois, Indiana, Kentucky, Western Tennessee and northern Mississippi, favoring prairie and pastureland habitat. This population is considered distinct based on a two-part coalescent analysis of five unlinked loci. Morphological description follows (Blanchard, 1921): 46–78 (60) dorsal blotches; ventral plates 196–215, (205); caudals 38–57, males 44–57 (51); females 38–52 (46); supralabials 7, sometimes 8; infralabials usually 9 often 10 occasionally 11; oculars 1 and 2; temporals normally 2 + 3 + 4; body slender and uniform; head scarcely distinct from the neck; snout longer and more pointed than in L. rhombomaculata; tail short and tapers rapidly. Lampropeltis calligaster contacts and may hybridize with Lampropeltis rhombomaculata in Northern Mississippi (Cook, 1945).


Distribution: Lampropeltis rhombomaculata is composed of eastern and southern populations in the state of Mississippi, favoring eastern dry pine and oak forests south to Louisianna, east to eastern Tennessee and the panhandle of Florida as well as on the Piedmont northwards along the eastern seaboard. This population is considered distinct based on this two-part coalescent-based analysis of five unlinked loci. Morphological description follows (Blanchard, 1921): 48–64 (55) dorsal blotches; ventral plates 191–213; caudals 31–55, males 37–55 (45) females 31–44 (41); supralabials 8 often 9; infralabials 8 often 9; oculars 1 and 2; temporals 2 + 3 + 4; body shape and pattern similar to that of L. calligaster. Lampropeltis rhombomaculata contacts and may hybridize with Lampropeltis calligaster in Northern Mississippi (Cook, 1945).


Type locality: South Florida. Holotype: FMNH 48265. Adult male from Okeechobee, Okeechobee County, Florida. Collected 31 May 1942 by Reid Paulk.

Distribution: Lampropeltis occipito-lineata is composed of all populations in South Florida and has been recorded from Brevard (Layne et al., 1986; Means, 1992), Desoto (Krysko and Hurt, 1998), Glades, Hendry (Krysko et al., 2000), Okeechobee (Layne et al., 1986; Means, 1992) and Osceola (Rushon, 2013) counties. This population is considered distinct based on a two-part coalescent analysis of five unlinked loci. Morphological description follows Price, 1987: Lampropeltis occipito-lineata is characterized by at least 78 distinct dorsal blotches, a complex network of dark lines on the back of the head, retention of the juvenile color pattern by adults, an obtuse canthus rostralis, a widening of the head in the temporal region and no more than 21 rows of dorsal scales.

Author contributions

A.D.M and F.T.B conceived the study; A.D.M generated sequence data and performed analysis. Both authors contributed to writing the paper.

Data accessibility

DNA sequences: Genbank accession numbers in Supplementary Material 2

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2016.09.006.

References


