

Niche divergence and lineage diversification among closely related *Sistrurus* rattlesnakes

J. A. WOOTEN* & H. L. GIBBS†‡

*Department of Biology, The University of Findlay, Findlay, OH, USA

†Department of Evolution, Ecology and Organismal Biology, Ohio State University, Columbus OH, USA

‡Ohio Biodiversity Conservation Partnership, Ohio State University, Columbus, OH, USA

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Abstract

Comparing niche divergence among closely related taxa can yield important insights into the ecological distinctiveness of genetically similar forms, and identify the processes that are responsible for diversification in such organisms. Here, we apply newly developed techniques for analysing niche divergence to assess how ecologically distinct a group of closely related rattlesnakes (*Sistrurus* sp.) are and to explore the role that niche divergence may have played in their diversification. We find that all taxa even the most recently evolved subspecies (approximately 100 000 years old) are now ecologically distinct, implying a role for ecology in the diversification process. Statistical analysis based on comparisons with null models show that niche divergence between forms is more common than niche conservation. Finally, there is nonlinear relationship between phylogenetic and niche divergence in this group whereby niche divergence develops more rapidly between recently diverged subspecies than more distantly related forms. Overall, our results argue that ecology may play an important role in the diversification process in these snakes.

Introduction

Analyses of niche relationships among closely related taxa can provide insights into the ecological distinctiveness and mechanisms responsible for the diversification of recently evolved taxa (Wiens & Graham, 2005; Raxworthy *et al.*, 2007; Schuller, 2009; McCormack *et al.*, 2010). Recent work in this area has focused on using spatially explicit environmental data analysed in the framework of environmental niche models (ENMs) to assess niche relationships among taxa (Kozak *et al.*, 2008). ENMs are used to generate geographic predictions of species' distributions based on environmental data from known location data (Peterson, 2001), and these spatial estimates of habitat suitability are then used as estimates of a species' niche (see discussion in Soberón, 2007). ENMs have been used to address important questions such as whether recently diverged taxa that show limited genetic differences are nonetheless ecologi-

cally distinct supporting the idea that they are separately evolving lineages (Rissler & Apodaca, 2007; Raxworthy *et al.*, 2007), and to examine the role that ecological differences have played as a mechanism influencing speciation in particular groups (Peterson *et al.*, 1999; Graham *et al.*, 2004; Knouft *et al.*, 2006; Kozak & Wiens, 2006; McCormack *et al.*, 2010).

With respect to the role of ecology in speciation, a key question is whether recently evolved taxa show evidence for niche divergence or niche conservation (Wiens & Graham, 2005). Evidence for divergence would support a role for ecological speciation in which divergent natural selection promotes diversification through adaptation to new environments (Schuller, 2001, 2009). On the other hand, evidence that niche characteristics are conserved across evolutionary time would suggest that ecological differences only accrue after speciation and suggests a different mechanism for the role of ecology in speciation namely that species evolve in allopatry under similar ecological conditions and later come into contact via range expansion at which time they develop niche differences (Price, 2008). ENMs have been widely used to examine these questions in a range of taxa with

Correspondence: H. Lisle Gibbs, Department EEOB, Ohio State University, 318 W. 12th Ave, Columbus, OH 43210-1293, USA. Tel.: +614 688 3861; fax: +614 292 2030; e-mail: gibbs.128@osu.edu

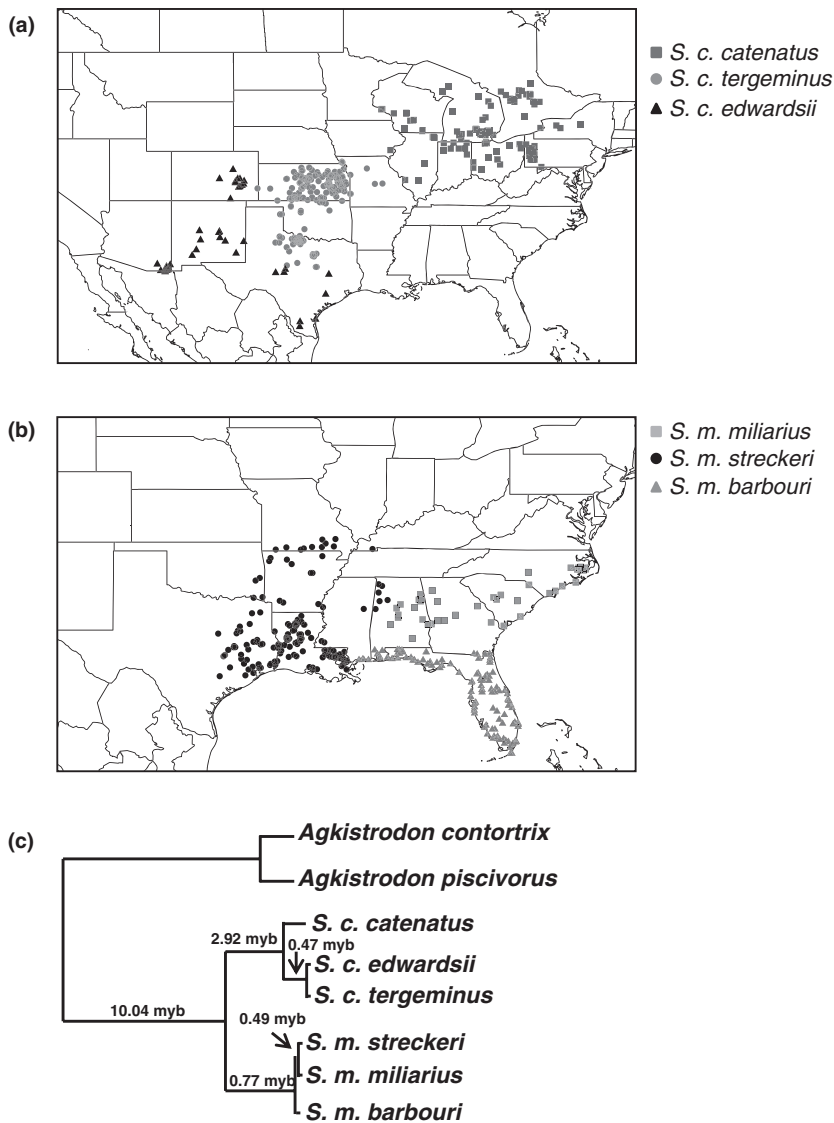


Fig. 1 Sample locations used to construct environmental niche models (ENMs) and estimated ranges for subspecies of (a) *Sistrurus catenatus* and (b) *S. miliarius* and (c) species tree based on multi-locus data showing phylogenetic relationships and estimated dates of divergence among all *Sistrurus* taxa. Estimated ranges in (a) and (b) were inferred from Campbell & Lamar (2004) whereas the phylogenetic tree in (c) is from Kubatko *et al.* (2011). Branch lengths are scaled according to expected number of substitutions at the TATA box-binding protein (TBP) locus (see Kubatko *et al.*, 2011).

evidence for both niche divergence and conservation (Peterson *et al.*, 1999; Graham *et al.*, 2004; Knouft *et al.*, 2006; & Wiens, 2006; Pyron & Burbrink, 2009; McCormack *et al.*, 2010). However, as pointed out by Pyron & Burbrink (2009), studies that examine the relative importance of these processes in wide-spread species complexes in the early stages of differentiation are still rare (but see McCormack *et al.*, 2010; Mao & Wang, 2011).

Reptiles are a promising group for applying ENMs to assess the impact of environmental factors on lineage diversification because, in general, they show limited dispersal and, hence, strong responses to local environmental variation (Burbrink *et al.*, 2008; Fontanella *et al.*, 2008; Chiucci & Gibbs, 2010; Row *et al.*, 2010). Furthermore, broadly distributed taxa in which phylogenetic

relationships are well established and where adaptive traits show interspecific variation are good candidates for the application of ENM approaches in assessing the role of ecology as an influence on lineage diversification. The massasauga and pigmy rattlesnakes (*Sistrurus* sp.) are one such group. *Sistrurus* rattlesnakes are a genera of New World pitvipers (Viperidae; Crotalinae) consisting of two named species: massasauga (*S. catenatus*) and pigmy (*S. miliarius*) rattlesnakes with each species consisting of three named subspecies (*S. c. catenatus*; *S. c. tergeminus*, and *S. c. edwardsii*, and *S. m. miliarius*, *S. m. barbouri*, and *S. m. streckeri*; Campbell & Lamar, 2004; Fig. 1). These snakes are found in a set of ecologically diverse habitats throughout North America (Conant & Collins, 1991; Fig. 1) and have been the focus of ecological and evolutionary studies (e.g. Farrell *et al.*, 1995; Sanz *et al.*,

2006; Gibbs & Rossiter, 2008; Gibbs & Mackessy, 2009; Kubatko *et al.*, 2011).

More specifically, Kubatko *et al.* (2011) conducted a phylogenetic analysis of the group in which they generated a species tree with branch lengths based on multiple gene trees, estimated dates of divergence, and conducted novel tests of the taxonomic distinctiveness of each subspecies. Despite limited differentiation between many subspecies, tests for lineage distinctiveness showed evidence for genetic distinctiveness of all subspecies, with the strongest support for the *S. c. catenatus* clade. Further, they observed that some of the geographic boundaries of distinct subspecies coincided with obvious biogeographic boundaries whereas others did not. This suggests that a diversity of speciation mechanisms, including ecological speciation, may have been responsible for diversification in this group. A role for ecological speciation is further supported by the fact that all subspecies show substantial differences in venom composition which is a key trait involved in prey capture and digestion and that these differences have functional consequences in terms of the ability to subdue ecologically diverse prey (Sanz *et al.*, 2006; Gibbs & Mackessy, 2009).

Here, we use a range of novel ENM-based techniques to examine patterns and mechanisms involved in speciation in this snakes. First, we use methods incorporated in ENMTools (Warren *et al.*, 2008) to assess the ecological distinctiveness of genetically similar subspecies of both species of *Sistrurus*. This addresses the question of whether these lineages, likely in the early stages of differentiation nonetheless, show evidence for niche divergence. Next, we use novel analytical techniques proposed by McCormack *et al.* (2010) (see also Arteaga *et al.*, 2011) to assess whether subspecies show evidence for niche divergence or conservation along multiple niche axis based on temperature, precipitation and habitat variables. These methods use an approach based on testing observed niche differences against null expectations to disentangle whether observed niche differences are real or are simply a consequence of spatial autocorrelation in environmental variation between the geographic regions over which the species are distributed (Godsoe, 2010). As described by McCormack *et al.* (2010), evidence for niche divergence requires that niche characteristics differ between species and that these differences are greater than background environmental divergence (see figure 1 in McCormack *et al.*, 2010). In contrast, niche conservation is supported when observed niche differences are smaller than background environmental differences. Finally, we assess how niche divergence varies with phylogenetic distance between individual taxa (Knouft *et al.*, 2006; Warren *et al.*, 2008). Evidence for a significant relationship between niche divergence and phylogenetic distinctiveness would support the idea that there is phylogenetic signal in the degree of niche overlap between taxa (Losos, 2008), and the shape of this relationship would suggest the rate at

which niche divergence has evolved through time in these snakes.

Methods

Occurrence and environmental data

To construct ENMs for *Sistrurus* rattlesnakes, we first downloaded occurrence data from natural history museum databases using HerpNet (<http://www.herpnet.org>) for each of six *Sistrurus* subspecies: *S. c. catenatus* (Eastern Massasauga), *S. c. tergeminus* (Western Massasauga), *S. c. edwardsii* (Desert Massasauga), *S. m. miliarius* (Carolina Pygmy Rattlesnake), *S. m. barbouri* (Dusky Pygmy Rattlesnake), and *S. m. streckeri* (Western Pygmy Rattlesnake). Localities with geographic coordinates were used without modification. Records with only detailed locality information were assigned coordinates using GEOLocate v. 3.0 (Tulane University Museum of Natural History; <http://www.museum.tulane.edu/geolocate/default.aspx>), a software program which georeferences natural history museum data. Localities were removed from the data set if they were assigned a question mark or if the projection was categorized as low coordinate accuracy. All localities were projected onto a map of North America using ArcGIS v. 9.3 and visually checked; any localities that seemed erroneous on the basis of existing distribution maps in Campbell & Lamar (2004) were removed. The downloaded occurrence data were an accurate representation of the geographic distribution of each *Sistrurus* subspecies (Fig. 1).

Construction of ENMs using Maxent

To generate environmental data for each location, we downloaded 19 climate variables representing measures and variability of temperature and precipitation (see Appendix S1 in Supporting Information) derived from 50 years (1950–2000) of climate data from WorldClim (<http://www.worldclim.org>; Hijmans *et al.*, 2005) and four land cover layers with data derived from satellite-borne remote sensors (NASA-MODIS/Terra data set; <http://edcimswww.cr.usgs.gov/pub/imswelcome/>) and radar scatterometers (Quick Scatterometer, QSCAT; Frohling *et al.*, 2006). The land cover layers included the normalized difference vegetation index (NDVI; measure of vegetative greenness); the yearly standard deviation of NDVI (NDVISTD); the QSCAT (measures reflected microwave radiation, and it is sensitive to slight variations in vegetation, canopy structure and moisture, and provides a measure of soil roughness and wetness in areas of sparse vegetation; Frohling *et al.*, 2006); and the percentage of tree cover (TREE). All layers used in the construction of the ENM were projected to the same spatial resolution (i.e. 1 km²) and geographic extent (i.e. United States and Canada).

All variables used to construct ENMs are defined in Appendix S1.

Seventy-five per cent of the occurrence points were randomly selected to construct the ENM using the 24 climate and vegetation variables for each subspecies in Maxent v. 3.3 (Phillips *et al.*, 2004, 2006). All remaining localities were retained for use as test data; this technique allows for direct comparison between the test and projected models. All point locality duplications were removed from the data set for ENM construction and subsequent statistical analyses. Only three climate and/or vegetation variables were highly correlated ($r > 0.95$); therefore, we decided to leave these variables in to construct the ENM; however, because of the possible interpretability issues associated with correlated variables, we subsequently performed principal component analysis (PCA), which converts possibly correlated variables into uncorrelated principal components (PCs). We then used the PCs in future statistical analyses. ENM predictions were converted to raster format and visualized in ArcGIS v. 9.3. Without absence data, the Maxent generated area under the curve (AUC) scores are difficult to interpret (Lobo *et al.*, 2008; Peterson *et al.*, 2008); therefore, we assessed the performance of the model using the minimum training presence value as calculated by Maxent. All probability of presence predictions less than or equal to the minimum training presence value as calculated by Maxent were then removed from further analyses. The predicted ENM of each *Sistrurus* subspecies was displayed in a single category in ArcGIS v. 9.3 and visualized.

Tests for niche overlap and environmental divergence

We used two approaches to test for niche overlap between the two *Sistrurus* species and among pair-wise combinations of *Sistrurus* subspecies. First, we used ENMTools (Warren *et al.*, 2008) to calculate Schoener's D (1968) and the Warren *et al.*'s I statistic (2008). These measures assign a numerical value that ranges from zero (i.e. no niche overlap) to one (i.e. identical niches; Warren *et al.*, 2008), and provide an indication of niche overlap for that comparison. We then used the background randomization test in ENMTools to test our null hypothesis that the observed niches and a taxon's background environment are similarly divergent. The background randomization test compares observed niche overlap values to a null distribution of 100 overlap values generated by comparing the ENM of one taxon to an ENM generated from randomly selected points from within the geographic distribution of the other taxon (Warren *et al.*, 2008). In order to obtain random points, we constructed a polygon that outlined the known geographic range for each taxon using Hawth's Tools in ArcMap v. 9.3. For each pairwise comparison, the number of randomly selected points was equal to the

sample size of the taxon from whose range the random points were drawn.

Second, for a more detailed analysis of any differences between specific environmental and land cover variables, we conducted a PCA in SPSS v. 17 (IBM Corporation, Somers, NY, USA) using occurrence point-specific environmental data extracted from DIVA-GIS v. 4.0 (WorldClim variables; Hijmans *et al.*, 2005) and ArcGIS v. 9.3 (land cover variables) and interpreted the importance of different variables to each factor based on their loading scores. A MANOVA with the PCA factor scores as the dependent variables and subspecies as the independent variables was conducted to compare overall niche differences. To limit the likelihood of a Type 1 error, we report pair-wise P -values with the critical value modified to control the false discovery rate (FDR; Narum, 2006).

Tests for niche conservatism and divergence

We followed the methodology of McCormack *et al.*, (2010) to determine patterns of niche conservatism or divergence for each subspecies. To determine whether two subspecies were more (= conservation) or less (= divergence) similar than expected based on differences in the environmental background in which they occur, we tested the null hypothesis that a species' niche was different from a random selection of background points. We used two approaches to do this: an analysis based on an overall niche model generated using Maxent and a more detailed assessment based on individual PC factors generated from environmental data. For the first analyses, we generated data for constructing null models using locality points and Hawth's Tools in ArcMap v. 9.3 to construct a polygon that represented the known geographic distribution for each subspecies. Random background points were then extracted from within the area represented by the polygon. The number of random points used in subsequent analyses was the same as the sample size of the taxon from whose range the random points were taken (McCormack *et al.*, 2010). The background randomization option in ENMTools (Warren *et al.*, 2008) compares the observed niche overlap values to a null distribution of 100 overlap values generated by comparing the ENM of one subspecies to an ENM created from the random background points from another subspecies, was used to test the null hypothesis (Warren *et al.*, 2008; McCormack *et al.*, 2010). Niche conservation or divergence was assessed by comparing the level of difference between observed niche overlap values (D_{obs}) with the null distribution of overlap values (D_{null}). Niche divergence (D) was inferred when $D_{\text{obs}} > D_{\text{null}}$, conservation (C) when $D_{\text{obs}} < D_{\text{null}}$ or neither (N) when $D_{\text{obs}} = D_{\text{null}}$.

For the second PC factor-based analyses, we used the methods described earlier to randomly select 1000 background points from within the constructed polygon that represented the geographic distribution for each

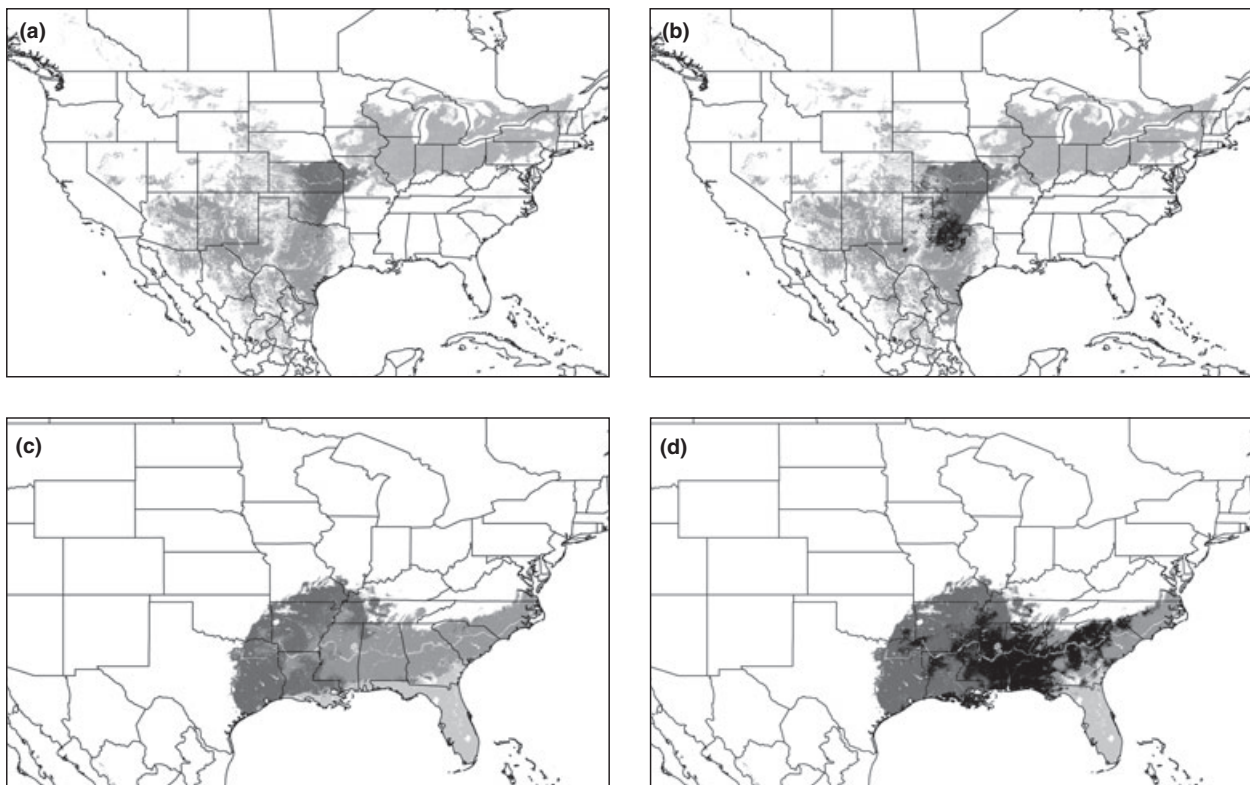


Fig. 2 Niche projections based on Maxent for (a) *Sistrurus catenatus*; (b) Subspecies of *S. catenatus*; (c) *S. miliarius*; and (d) Subspecies of *S. miliarius*. Overlap between projections is indicated by dark shading.

subspecies. Environmental data were extracted for these background points using DIVA-GIS v. 4.0 (Hijmans *et al.*, 2001) and ArcMap v. 9.3. PCA was conducted to reduce the number of variables. The PCA factor scores from the first five axes consistently explained > 90% of the overall variance (each axis contributed at least 3% of the variance) and were used as the observed niche values in comparisons with background points. Niche conservation or divergence was assessed by comparing the level of difference between the mean observed (i.e. observed niche values) scores and a 95% confidence intervals for distribution based on background PCA factor scores. Following McCormack *et al.* (2010) *D* or *C* values were considered to be statistically significant when they satisfied two criteria: first, the mean observed PC factor score was outside the 95% CI of the null distribution, and paired *t*-tests showed significant differences in the observed and null distributions.

Phylogenetic divergence and niche overlap

To assess the relationship between niche divergence and phylogenetic divergence, for each pairwise combination of subspecies ($n = 12$ comparisons) we calculated patristic distances based on the species tree for the group generated by Kubatko *et al.* (2011) and also determined

observed niche overlap in terms of *I* and *D* values using ENMTools. We then used a Mantel test to determine whether there was a significant association between distances and overlap values. To assess the shape of the relationship, we compared the amount of variation explained by equations with and without nonlinear

Table 1 upper matrix (*D*) and lower matrix (*I*) values for *Sistrurus* calculated using ENMTools (Warren *et al.*, 2008). A indicates species-level differences, and B indicates subspecies-level differences.

A		<i>catenatus</i>	<i>miliarius</i>	
		species	species	
<i>S. catenatus</i> species	1.000	0.424		
<i>S. miliarius</i> species	0.136	1.000		
B				
		<i>S. c. catenatus</i>	<i>S. c. tergestinus</i>	<i>S. c. edwardsii</i>
<i>S. c. catenatus</i>	1.000	0.365	0.347	
<i>S. c. tergestinus</i>	0.063	1.000	0.466	
<i>S. c. edwardsii</i>	0.037	0.179	1.000	
		<i>S. m. miliarius</i>	<i>S. m. streckeri</i>	<i>S. m. barbouri</i>
<i>S. m. miliarius</i>	1.000	0.609	0.388	
<i>S. m. streckeri</i>	0.414	1.000	0.417	
<i>S. m. barbouri</i>	0.102	0.144	1.000	

terms using adjusted r -squared values that account for the number of terms in different equations. Statistical evaluation of the amount of variation explained by different equations was not possible because of the nonindependence of the individual data points.

Results

ENMs for *Sistrurus* subspecies

We obtained 1312 occurrence points for *S. m. barbouri* ($n = 254$), *S. m. miliarius* ($n = 60$), *S. m. streckeri* ($n = 255$), *S. c. catenatus* ($n = 339$), *S. c. edwardsii* ($n = 81$), and *S. c. tergeminus* ($n = 323$; Fig. 1). Predicted distributions of each subspecies based on analysis of the environmental data associated with these points using Maxent are shown in Fig. 2. These projections represent a spatially explicit estimation of habitat suitability using information from all environmental variables that were considered. In all cases, the AUC in the Maxent analysis was well above random (i.e. AUC = 0.50), with an AUC range from 0.990 to 0.998 (Appendix S2). The predicted niche models for each of the *Sistrurus* subspecies are in close agreement with the broad outlines of the current known geographic distributions of each lineage (Fig. 2; Campbell & Lamar, 2004); however, they fail to capture the patchy nature of the contemporary distributions of

some subspecies (e.g. *S. c. catenatus*) likely due to the impact of recent anthropogenic impacts on the distributions of these snakes (Chiucchi & Gibbs, 2010).

To determine the variables that were most important in ENM construction, we used the output from Maxent to determine the relative contributions of the climate and land cover variables to the ENM predictions (Appendix S3). It appears that temperature is one of the most important drivers of the ENM predictions for both species, with precipitation playing a larger role in the predicted distribution for *S. miliarius* compared with *S. catenatus*. For the *S. catenatus* subspecies, only BIO10 (mean temperature of the warmest quarter) overlapped in the prediction for *S. c. tergeminus* with *S. c. catenatus* and BIO8 (mean temperature of the wettest quarter) overlapped for *S. c. tergeminus* with *S. c. edwardsii*. For the *S. miliarius* subspecies, only BIO18 (precipitation of warmest quarter) overlapped in the prediction for *S. m. miliarius* with *S. m. barbouri*. This pattern of variable overlap suggests that the ecological requirements are different among and within *S. catenatus* and *S. miliarius* subspecies.

Tests of niche overlap

Comparisons of D and I values based on the overall ENMs are all significantly lower than expected from a random

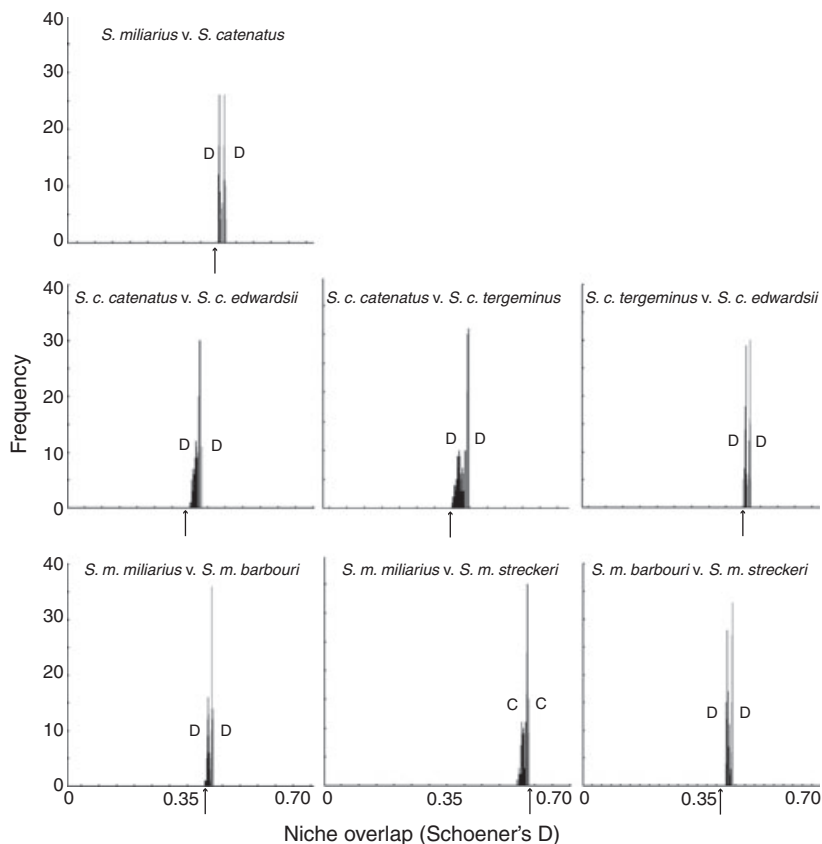


Fig. 3 Frequency histograms from tests of niche conservatism and divergence from an analysis of observed and 'null' niche models using ENTools. The arrows represent the niche overlap value compared with the background divergence. Each histogram represents a pairwise comparison between two listed taxa, where the environmental niche models (ENM) for taxon A is compared with the background points from taxon B and vice versa. Niche overlap values (indicated by an arrow) that are smaller than the null distribution of background divergence support niche divergence (D). Niche conservatism (C) is supported when the niche overlap values are larger than the null distribution.

distribution which indicates that both species and each subspecies within each species are ecologically distinct lineages (Table 1). Specifically, there was relatively moderate niche overlap at the species level for *S. catenatus* and *S. miliarius* ($D = 0.424$; $I = 0.136$; Table 1). For the *S. catenatus* subspecies, the greatest amount of niche overlap was exhibited by *S. c. tergeminus* and *S. c. edwardsii* ($D = 0.466$; $I = 0.179$; Table 1). For the *S. miliarius* subspecies, there was considerable overlap between *S. m. streckeri* and *S. m. miliarius* ($D = 0.609$; $I = 0.414$), but only moderate overlap between *S. m. barbouri* and *S. m. miliarius* ($D = 0.388$; $I = 0.102$) and *S. m. barbouri* and *S. m. streckeri* ($D = 0.417$; $I = 0.144$; Table 1).

To identify specific climate and vegetation variables that best explained the geographic ranges occupied by each lineage, we conducted a PCA using point-specific environmental data extracted from DIVA-GIS v. 4.0 (Hijmans *et al.*, 2001) and ArcGIS v. 9.3. We found that the first five PCs explained 89.23% of the variance, with 46.64%, 23.16%, 8.60%, 6.34%, and 4.50% for factors 1–5, respectively. Temperature explained most of the variation (i.e. PC factor 1) observed in *Sistrurus*, especially the annual temperature range (BIO7), temperature seasonality (BIO4), minimum temperature of the coldest month (BIO6), and mean temperature of the coldest

quarter (BIO11). In order to confirm that there were substantial environmental differences among the lineages, we completed a MANOVA using the PCA factor scores as the dependent variables and nominal subspecies as the independent variables. We found that there was an overall significant difference in the factor scores ($F_{1,25} = 560.449$; $P < 0.0001$). We found significant differences in the factor scores, suggesting that there are abiotic and vegetation differences among subspecies. Specifically, for PC factors 1, 2 and 3, all subspecies were significantly different ($P \leq 0.0001$) from each other except *S. m. miliarius* compared with *S. m. streckeri* ($P = 0.252$; $P = 1.000$; $P = 1.000$ for factors 1, 2 and 3, respectively); for factor 4, all subspecies were significantly different ($P \leq 0.0001$) from each other except *S. c. catenatus* and *S. c. edwardsii* ($P = 0.052$). Finally, for factor 5, *S. m. barbouri* and *S. m. miliarius* ($P = 0.103$), *S. m. streckeri* and *S. m. streckeri* ($P = 1.000$) were the only pair-wise combinations of subspecies not significantly different at $P \leq 0.0001$ level.

Niche conservatism and divergence

Results of the background randomization tests using ENMTools (Warren *et al.*, 2008) suggest that niche

Table 2 Summary of niche assessment for conservatism (*C*) and divergence (*D*) using mean background differences in principal component analysis factor scores for climate/vegetation data for *Sistrurus*. Values for factor scores are given as mean \pm standard error. Values in parentheses represent null distributions between species' respective geographic ranges based on background divergence. If the null distribution is larger than the niche value, then the niche is considered divergent; if smaller, the niche is considered conserved.

	PC factor 1	PC factor 2	PC factor 3	PC factor 4	PC factor 5
Pair-wise comparison					
<i>S. catenatus</i> sp. vs.	6.000 \pm 0.001 <i>D</i>	0.812 \pm 0.002 <i>D</i>	0.370 \pm 0.001 <i>C</i>	0.127 \pm 0.001 <i>C</i>	0.126 \pm 0.001 <i>D</i>
<i>S. miliarius</i> sp.	(5.802, 5.862)	(0.383, 0.592)	(0.399, 0.484)	(0.309, 0.421)	(0.049, 0.059)
<i>S. c. catenatus</i> vs.	8.086 \pm 0.002 <i>D</i>	0.089 \pm 0.002 <i>D</i>	0.473 \pm 0.002 <i>D</i>	0.364 \pm 0.002 <i>D</i>	0.402 \pm 0.001 <i>D</i>
<i>S. c. edwardsii</i>	(7.242, 7.514)	(0.038, 0.0478)	(0.221, 0.345)	(0.179, 0.182)	(0.189, 0.243)
<i>S. c. catenatus</i> vs.	5.661 \pm 0.001 <i>D</i>	0.056 \pm 0.001 <i>C</i>	0.415 \pm 0.002 <i>C</i>	0.308 \pm 0.001 <i>C</i>	0.648 \pm 0.001 <i>D</i>
<i>S. c. tergeminus</i>	(5.427, 5.533)	(0.659, 0.877)	(0.899, 0.914)	(0.336, 0.364)	(0.238, 0.451)
<i>S. c. edwardsii</i> vs.	5.978 \pm 0.004 <i>D</i>	0.914 \pm 0.015 <i>D</i>	1.342 \pm 0.002 <i>D</i>	0.154 \pm 0.003 <i>D</i>	0.168 \pm 0.002 <i>D</i>
<i>S. c. tergeminus</i>	(4.875, 4.945)	(0.582, 0.784)	(1.131, 1.228)	(0.045, 0.129)	(0.010, 0.119)
<i>S. m. barbouri</i> vs.	6.720 \pm 0.004 <i>D</i>	0.935 \pm 0.005 <i>D</i>	1.074 \pm 0.005 <i>D</i>	0.632 \pm 0.005 <i>D</i>	0.411 \pm 0.005 <i>D</i>
<i>S. m. miliarius</i>	(5.604, 5.948)	(0.786, 0.845)	(0.127, 0.246)	(0.537, 0.540)	(0.384, 0.391)
<i>S. m. barbouri</i> vs.	5.687 \pm 0.002 <i>D</i>	0.091 \pm 0.002 <i>C</i>	0.875 \pm 0.002 <i>D</i>	0.055 \pm 0.001 <i>C</i>	0.052 \pm 0.001 <i>C</i>
<i>S. m. streckeri</i>	(5.603, 5.653)	(0.756, 0.812)	(0.364, 0.487)	(0.399, 0.426)	(0.149, 0.152)
<i>S. m. miliarius</i> vs.	0.584 \pm 0.005 <i>C</i>	1.008 \pm 0.006 <i>D</i>	1.167 \pm 0.006 <i>C</i>	0.479 \pm 0.007 <i>C</i>	1.651 \pm 0.003 <i>D</i>
<i>S. m. streckeri</i>	(0.951, 1.125)	(0.661, 0.775)	(2.085, 2.145)	(1.215, 1.428)	(0.771, 0.995)
Variance explained (%)	47.138	23.068	8.832	6.590	4.363
Top four variable loadings	BIO7 BIO4 (BIO6) (BIO11)	BIO7* (BIO14) (BIO17) (BIO19)	BIO11* BIO4 (NDVISTD)* (BIO18)	BIO16* BIO13 (NDVI)* (NDVISTD)	BIO13* BIO11 (BIO8) (QSCAT)
Biological interpretation	Temp	Temp/seasonal precip	Complex†	Precip/greenness	Precip/temp

For variable description, see Appendix S1.

Bold font indicates significant niche differences compared with background points and with the independent samples *t*-test, and parentheses indicate variable loadings with eigenvector with opposite sign.

*Variables with high contribution to a given PC axis; next eigenvector is at least 0.1 greater.

†Complex (temp/seasonal greenness/precip).

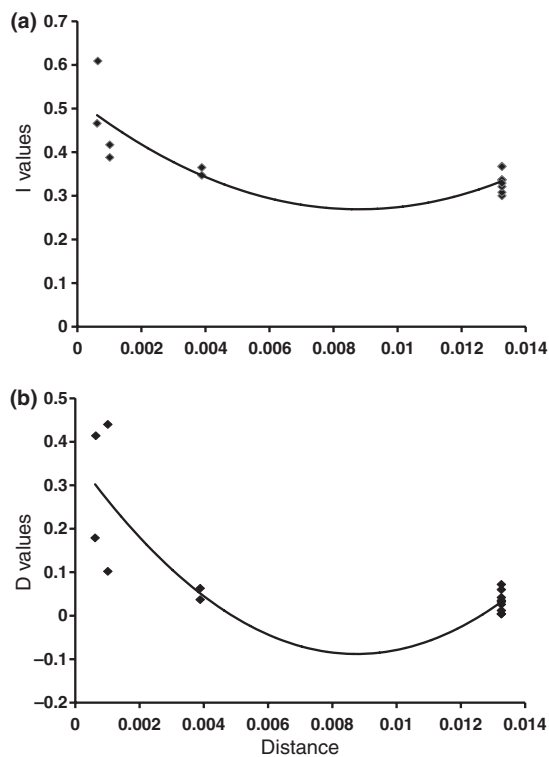


Fig. 4 Relationship between patristic distance and niche overlap values for all pairwise comparisons of *Sistrurus* taxa. Plots are shown for (a) Schoener's D and (b) Warren *et al.*'s I statistic. Line shows the best-fit line for either $-1/D$ or $-1/I$ and patristic distance.

divergence is by far the most common pattern observed in these snakes when assessed using overall niche model characteristics (Fig. 3). Six of seven comparisons show significant divergence in overall niche characteristics; in that, observed D values were less than D values from null distributions for pair-wise comparisons of specific taxa. The single exception was a pair of sister taxa within the *S. miliaris* clade (*S. m. miliaris* and *S. m. streckeri*), which showed significant evidence for niche conservation.

Most individual PC factors showed evidence for statistically significant niche divergence (D) or conservation (C) in most comparisons (Table 2). The overall patterns across different taxonomic groupings are summarized in Table 3. In general, niche divergence was more commonly observed than niche conservation. For example, for the two 'major' PC factors (1 and 2) which explain more than 80% of environmental variation, divergence was the most commonly observed result for comparisons at the species level (100% of all factor comparisons), those involving both *S. catenatus* and *S. miliaris* subspecies (50%), and for *S. catenatus* subspecies only (67%; Table 3). The exception was comparisons involving *S. miliaris* subspecies only where factors showing evidence for divergence and conservation were equally frequent. Similar patterns are observed when results from the 3 'minor' PC factors which

Table 3 Summary of niche divergence analysis based on PC factor for different taxonomic levels in *Sistrurus*. D = number of comparisons of PC factors showing statistical evidence for niche divergence based on both comparisons with null distributions and t -tests; C = number of comparisons showing statistical evidence for niche conservatism; N = number of comparisons that did not meet the statistical criteria for significant D or C values; Total: total number of comparisons made for given factors within a particular taxonomic level.

Taxonomic level	Niche divergence pattern	Major factors (PC 1 and 2; % total)	All factors (PC 1–5; % total)
Species-level only	D	2 (100)	3 (60)
	C	0	2 (40)
	N	0	0
	Total	2	6
All subspecies	D	6 (50)	17 (57)
	C	3 (25)	10 (33)
	N	3 (25)	5 (10)
	Total	12	32
<i>S. catenatus</i> subspecies	D	4 (67)	11 (73)
	C	1 (17)	3 (20)
	N	1 (17)	1 (7)
	Total	6	15
<i>S. miliaris</i> subspecies	D	2 (33)	6 (40)
	C	2 (33)	7 (47)
	N	2 (33)	2 (13)
	Total	6	15

account for < 20% of overall variation are combined with the results described earlier for PC factors 1 and 2 (Table 3). In summary, patterns of niche divergence are more common than conservation in this group although there is evidence of clade age having a possible effect on this pattern with the older *S. catenatus* clade (> 2.9 MYR bp – see Kubatko *et al.*, 2011), showing more evidence for divergence than the more recently evolved *S. miliaris* subspecies (> 0.77 MYR bp).

Niche overlap relative to phylogenetic divergence

There was a significant negative relationship between phylogenetic distance and both D and I values for pairwise comparisons of all *Sistrurus* subspecies (Mantel test; D : $r = -0.71$; $P < 0.001$; I : $r = -0.71$; $P < 0.005$). The relationship is nonlinear with a disproportionately rapid decline in overlap for closely related taxa that then plateaus out for more distantly related forms (Fig. 4). This inference is supported by the observation that the adjusted r -squared value for a regression of the form ($-1/D$ or I) is higher ($\geq 60\%$) than when a simple linear relationship is fit to the data ($\leq 47\%$; Fig. 4). The interpretation is that this pattern is consistent with a temporal sequence in which there is an initial burst of divergence among newly speciating forms that reaches a roughly constant level of overlap among more diverged taxa.

Discussion

Ecological distinctiveness of recently evolved taxa

A significant result of our study is that multiple analyses show strong evidence for ecological distinctiveness of all *Sistrurus* taxa, even recently evolved subspecies with minimal genetic differentiation that may represent incipient species. This has two implications. First, the lack of ecological exchangeability between lineages is independent evidence that these lineages are independent and distinct (Raxworthy *et al.*, 2007; Rissler & Apodaca, 2007; Leaché *et al.*, 2009). Regarding the first point, Kubatko *et al.* (2011) showed that with the exception of *S. c. catenatus*, all other subspecies originated ≤ 0.77 MYR bp and that the evidence for the presence of separate lineages through lineage distinctiveness tests based on genetic data was limited. It was unclear whether this lack of support for separate lineages was due to a true lack of differentiation or was a consequence of the limited sample sizes used in the analysis and/or high levels of retained ancestral polymorphism. As pointed out by Leaché *et al.* (2009), recent radiations involve a temporally extended process whereby populations may diverge genetically, morphologically, or ecologically, and these properties of species provide the operational criteria useful for species delimitation. Coincident differentiation in multiple criteria provides stronger support for lineage distinctiveness (Reeves & Richards, 2011). Our documentation of ecological differentiation combined with previous evidence for morphological differentiation (e.g. Gloyd, 1940; but see Gibbs *et al.*, 2011) supports the idea that these subspecies are independently evolving lineages. As a consequence, this suggests that the lack of phylogenetic distinctiveness in multiple loci in most of the subspecies is likely due to limited sampling and/or ancestral polymorphism; hence, that ecological distinctiveness in this group develops early in lineage formation before strong phylogenetic distinctness in single-locus nuclear loci is apparent.

We can also gain insights into the specific traits that may underlie adaptive differentiation between subspecies by identifying the specific ecological variables in the ENMs that contribute most strongly to ecological differentiation between lineages, although this is limited by the range of variables used in the analyses. In our analysis, we included two broad classes of environmental variables: temperature and precipitation- and vegetation-related variables (Appendix S1). Examining the relative contributions of each class of variables in both the Maxent-based ENM and the PC-based analyses, both show that the temperature and precipitation variable are far more important in explaining ecological differences between snake lineages than are vegetation-based variables (e.g. Table 2). This is consistent with the idea that adaptations in sedentary ectotherms such as these snakes may be in terms of physiological adaptations

related to water use and/or temperature tolerance (Porter & Gates, 1969). Such adaptations have not been documented in *Sistrurus* but may be common in reptiles. What have been documented are differences in venom composition (Sanz *et al.*, 2006; Gibbs & Mackessy, 2009) which are presumably related to differences in prey consumed by different subspecies (e.g. Daltry *et al.*, 1996). Variation in prey communities is more difficult to link to variation in the temperature and precipitation variables that appear to explain ecological differences between subspecies, especially given a lack of a contribution by vegetation-related variables that would seem to be more related to habitat differences that could influence prey animal community composition. This reflects a general weakness of the ENM approach as currently implemented in many studies which is that the environmental variables used to construct the models are limited and may only reflect certain aspects of the overall niche of a given taxon (Soberón, 2007; Kozak *et al.*, 2008).

Niche divergence and speciation

The application of novel techniques (Warren *et al.*, 2008; McCormack *et al.*, 2010) for inferring whether divergence between *Sistrurus* taxa is associated with niche conservation or divergence signals a significant role for divergence at all stages of lineage formation in these snakes. These approaches represent a significant advance because they take into account possible spatial autocorrelation in environmental variables between the regions where the taxa being examined are distributed (e.g. Costa *et al.*, 2007; Godsoe, 2010) and hence offer stronger support for claims that observed patterns represent meaningful patterns of divergence and are not a sole consequence of taxa occupying different geographic locations. Based on these approaches, the over-riding importance of divergence between some pairs of sister taxa (e.g. *S. c. tergeminus* and *S. c. edwardsii*) and more distantly related sets of taxa argues that ecological differentiation may play an important role in both the origin and ongoing development of species in this closely related group of reptiles. This conclusion is tempered by the requirement that patterns of niche divergence between sister taxa are required for direct inferences about the role that ecology plays in the origin of lineages (McCormack *et al.*, 2010). Due to the small size of this clade of snakes, we only have two such comparisons one of which shows mainly evidence for divergence whereas the other for conservation. However, divergence can also develop following secondary contact of lineages that have initially become reproductively isolated for reasons other than ecology such as allopatric isolation (Price, 2008). Although the precise role that divergence plays in each temporal stage of lineage formation remains to be determined, overall our results suggest these snakes may represent another example in ectothermic vertebrates

where analyses based on ENMs suggest that ecological speciation plays a significant role in lineage generation (Graham *et al.*, 2004; Raxworthy *et al.*, 2007).

We also note that there is evidence for a limited role for niche conservatism in these snakes: one pair of sister taxa (*S. m. miliarius* and *S. m. streckeri*) showed a pattern of conservatism based on overall ENM analyses and in the PC-based analyses various combinations of taxa exhibited conservatism along different factor axes. We note that these taxa have geographic ranges which meet close to the Mississippi River which is consistent with niche conservatism between lineages being observed when ranges are separated by a feature of the landscape which could represent a significant biogeographic barrier (Peterson *et al.*, 1999). Niche conservatism has been demonstrated to play a significant role in species formation in other ectotherms such as North American *Plethodon* salamanders (Kozak & Wiens, 2006; Shepard & Burbrink, 2008, 2009). Less common are taxa that show a pattern of mixed evidence for divergence and conservatism, but this pattern has been found for another snake species, the common kingsnake (*Lampropeltis getula*) by Pyron & Burbrink (2009). However, we stress that overall our results argue for a dominant role for divergence in this group.

Finally, the significant relationship between phylogenetic divergence and levels of niche overlap is evidence that there is phylogenetic signal in niche relationships among *Sistrurus* taxa and contrasts with results from other ectotherms such as *Anolis* lizards in which no such relationship was found (Knouft *et al.*, 2006; Warren *et al.*, 2008). Superficially, such a relationship could be interpreted as evidence for phylogenetic niche conservatism but, as discussed by Losos (2008), while such a relationship is necessary to infer niche conservatism, it is not sufficient. Rather, comparison with the degree of divergence expected under some sort of null model whereby levels of divergence evolve due to genetic drift or fluctuating selection is required with the expectation that conservatism would be inferred if levels of divergence are less than the null expectation. We did not conduct such an analysis due to the small size of our data set.

We see that such comparisons can provide insights into another phenomenon, namely the rate at which divergence develops between lineages. The inverse relationship between overlap and phylogenetic distance suggests that initially, ecological divergence between taxa develops rapidly before levelling off when overlap is compared between increasingly divergent forms. This is consistent with a significant role for selection for niche divergence in the early stages of lineage formation that drives a rapid decline in overlap followed by a decline in the strength of this selection as divergence develops. Clearly, this process cannot explain patterns of divergence of all lineages in these snakes (e.g. *S. m. miliarius* and *S. m. streckeri*), but where niche divergence is implicated, this pattern implies that ecological speciation may be more important in

lineage formation rather than as a force in subsequent divergence once a baseline level of niche divergence has been reached. However, we emphasize that models that examine the relationship between phylogenetic signal and neutral and selection-driven evolutionary processes demonstrate that different processes can produce similar results in terms of patterns of signal (Revell *et al.*, 2008). This raises the possibility that alternate evolutionary processes other than divergence via selection could also contribute to the nonlinear relationship between divergence and patristic distance.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Climate and vegetation variables and descriptions used to construct ecological niche models (ENM).

Appendix S2 Logistic threshold values and the area under the curve (AUC) to assess niche model performance for each *Sistrurus* subspecies derived by Maxent v. 3.3.

Appendix S3 Relative contributions of the climate and land cover variables to the ENM predictions.

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