ORIGINAL ARTICLE

Origin of a cryptic lineage in a threatened reptile through isolation and historical hybridization

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Identifying phylogenetically distinct lineages and understanding the evolutionary processes by which they have arisen are important goals of phylogeography. This information can also help define conservation units in endangered species. Such analyses are being transformed by the availability of genomic-scale data sets and novel analytical approaches for statistically comparing different historical scenarios as causes of phylogeographic patterns. Here, we use genomic-scale restriction-site-associated DNA sequencing (RADseq) data to test for distinct lineages in the endangered Eastern Massasauga Rattlesnake (*Sistrurus catenatus*). We then use coalescent-based modeling techniques to identify the evolutionary mechanisms responsible for the origin of the lineages in this species. We find equivocal evidence for distinct phylogenetic lineages within *S. catenatus* east of the Mississippi River, but strong support for a previously unrecognized lineage on the western edge of the range of this snake, represented by populations from Iowa, USA. Snakes from these populations show patterns of genetic admixture with a nearby non-threatened sister species (*Sistrurus tergeminus*). Tests of historical demographic models support the hypothesis that the genetic distinctiveness of Iowa snakes is due to a combination of isolation and historical introgression between *S. catenatus* and *S. tergeminus*. Our work provides an example of how model-based analysis of genomic-scale data can help identify conservation units in rare species.

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INTRODUCTION

The capacity to produce genomic-scale data sets from nonmodel organisms has transformed our ability to characterize patterns of genetic diversity within species and understand the historical processes that generate such patterns (Eklom and Galindo, 2011). High-throughput sequencing technologies, novel cost-effective methods for constructing reduced representation libraries (that is, restriction site-associated DNA sequencing (RADseq); Davey and Blaxter, 2011; Davey *et al.*, 2011) and the development of statistical approaches that allow historical demographic analyses of these data (that is, Gutenkunst *et al.*, 2009; Excoffier *et al.*, 2013; Leaché *et al.*, 2014) are important factors contributing to this transformation. These novel genomic-scale data sets can reveal cryptic genetic diversity within recognized taxa, estimate demographic parameters characterizing populations and identify important recent and historical events that have shaped existing genetic diversity, such as hybridization between lineages (Luikart *et al.*, 2003; Ellegren, 2013). Such information can inform decisions about how to designate conservation units in rare taxa and, as a result, genomic-scale analyses now play an important role in conservation genetic research (Avice, 2010; Funk *et al.*, 2012; Narum *et al.*, 2013).

The Eastern Massasauga Rattlesnake (*Sistrurus catenatus*) is a snake of conservation concern that occurs in small, isolated populations across eastern North America. It is designated as threatened or endangered in every US state and Canadian province in which it occurs (Szymanski, 1998), and is currently being considered for listing as a federally endangered species under the Endangered Species Act in the United States (United States Fish and Wildlife Service, 2015).

Genetic studies to date have drawn different conclusions as to whether distinct phylogenetic lineages that would represent evolutionary significant units under the Endangered Species Act occur in this species. For example, Kubatko *et al.* (2011) found no evidence for phylogeographic structure based on a multilocus analysis of 20 nuclear DNA markers, although the number and geographic range of samples analyzed was limited. In contrast, Ray *et al.* (2013) analyzed a much larger and more widespread set of samples with a single mitochondrial DNA (mtDNA) locus and identified three geographically separate mtDNA lineages (Western, Central and Eastern). These designations are currently being considered for use in a captive breeding program for this species (Ray *et al.*, 2013). However, given that this inference is based on a single mtDNA locus, it is unclear whether this pattern represents variation across the genome as a whole, and hence whether it provides an accurate representation of the phylogenetic history of this species.

Both of the studies highlighted above were focused on inferring patterns of phylogenetic diversity within *S. catenatus*. However, recent analytical advances that use the site frequency spectrum (SFS) generated from genomic-scale data sets provide opportunities to also assess the evolutionary and ecological mechanisms that generate observed phylogeographic patterns (Gutenkunst *et al.*, 2009; Excoffier *et al.*, 2013). In this study, we use a genomic-scale single-nucleotide polymorphism (SNP) data set that includes samples collected from across the range of *S. catenatus* to reexamine whether important phylogenetic lineages are present within this species. We do this by first using clustering algorithms to identify major genetic groups within *S. catenatus*, and

then use species tree-based phylogenetic methods to test for significant phylogenetic differentiation between these groups. We then apply recently described likelihood-based methods that use the SFS to test various historical demographic scenarios as the cause of the observed patterns of diversity, and to estimate associated demographic parameters. We discuss the implications of our results in terms of how they contribute to the designation of conservation units within *S. catenatus*.

MATERIALS AND METHODS

Samples and sequencing methods

We obtained blood or tissue samples for *S. catenatus* individuals from 35 populations spanning the geographic range of this species ($N=1-13$ per population), and for an additional eight *Sistrurus tergisus* individuals (Figure 1 and Table 1). We also obtained one *Sistrurus miliarius* sample that was used to polarize SNPs when generating unfolded SFSs. Genomic DNA was extracted using either DNA Blood and Tissue Kits (Qiagen, Valencia, CA, USA) or phenol-chloroform. Double-digest RADseq libraries (Peterson *et al.*, 2012) were generated with *EcoRI* and *SbfI* restriction enzymes (New England Biolabs, Ipswich, MA, USA) and a modified version of the RADseq protocol described in DaCosta and Sorenson (2014). Details of the library prep methods are included in the Supplementary Information. Pooled libraries of up to 36 individuals were run in single-end 50 bp runs on either entire lanes of an Illumina GAI (San Diego, CA, USA) sequencer or as partial lanes (ranging from 10 to 25%, depending on the number of individuals in the specific library) on an Illumina HiSeq 2500 sequencer.

Bioinformatic methods

De novo locus assembly, SNP identification and genotyping were carried out on the raw fastq data using AftRAD version 4.1 (Sovic *et al.*, 2015) that efficiently assembles RADseq loci while accounting for indel variation. Two separate AftRAD analyses were performed. The first analysis generated data that were used for the clustering and species tree analyses, and included a maximum of three individuals per population (see Table 1). Based on results from these analyses, we performed a second AftRAD run to generate a separate data set for demographic modeling analyses. This run only included *S. catenatus* samples from Buhr Access, Iowa and Killdeer Plains, Ohio ($N=13$ each), samples from *S. tergisus* and one *S. miliarius* individual that was used to polarize SNPs

when building an unfolded SFS. Details on the parameter settings for these AftRAD runs are provided in the Supplementary Information.

Genetic clustering analyses

We performed clustering analyses on the genetic data using two programs: Structure (Pritchard *et al.*, 2000) and adegenet (Jombart and Ahmed, 2011). The purpose of these analyses was to identify major genetic groups that could be subsequently tested for distinctiveness with phylogenetic methods. In both analyses we used a maximum of three samples per population to reduce possible effects of unequal sample sizes on clustering results (Kalinowski, 2011). Structure uses a Bayesian approach to cluster samples into groups that most closely meet Hardy-Weinberg equilibrium expectations, and runs were performed for K values ranging from 1 to 6. Three independent runs were completed for each K value, with 2×10^5 Markov chain Monte Carlo iterations, plus a 10% burn-in period, per run. Optimal K values were identified using the ΔK method of Evanno *et al.* (2005) as calculated using Structure Harvester (Earl and Vonholdt, 2012).

We also identified genetic clusters using the find.clusters function in adegenet 1.3–9.2 (Jombart and Ahmed, 2011) that uses a model-free K -means clustering approach to identify distinct groups and, thus, unlike Structure, does not rely on assumptions of Hardy-Weinberg equilibrium and linkage equilibrium within clusters. As for the Structure runs, optimal clustering solutions were generated for K values ranging from 1 to 6, with clustering solutions at each k value evaluated based on Bayesian information criterion scores. Finally, to summarize how variation is partitioned among the identified groups, we performed a discriminant analysis of principal components (Jombart *et al.*, 2010) based on the optimal clustering solution identified in adegenet, and used Arlequin v3.5 (Excoffier and Lischer, 2010) to calculate F_{ST} values between each identified cluster.

Species tree analyses

Given the results from the clustering analyses described above, we used two approaches to test for significant phylogenetic differentiation among the major genetic groups identified within *S. catenatus*. First, we performed species tree phylogenetic analyses using SNAPP (Bryant *et al.*, 2012). Because the computational demands of SNAPP depend on the number of individuals included in the analysis (Bryant *et al.*, 2012), we used a subsampling approach similar to that used by Harvey and Brumfield (2015) in which we randomly selected three individuals (six chromosomes) to represent each of the groups

Figure 1 Map of sampling locations for *S. catenatus* samples (darker shading) and *S. tergisus* samples (lighter shading). Populations are indicated with smaller bold font, and state or province names are indicated with larger bold font. See Table 1 for population abbreviations and sample sizes.

Table 1 Sampling locations with location codes for *Sistrurus catenatus* and *Sistrurus tergeminus* included in the study

Location	N, total	N, clustering	N, fastsimcoal
<i>Illinois</i>			
Carlyle Lake (CARL)	3	3	
Cook County (COOK)	1	1	
Eldon Hazlet State Park (EHSP)	2	2	
Ryerson (RYSN)	2	2	
South Shore State Park (SSSP)	3	3	
Willow Sanders (WLSN)	2	2	
<i>Indiana</i>			
Dunlap (DNLP)	3	3	
<i>Iowa</i>			
Buhr Access (BUHR)	13	3	13
Chickasaw (CKSW)	2	2	
Sweet Marsh (SWTM)	1	1	
<i>Kansas</i>			
Ellsworth County (TERG)	4	2	4
Russell County (TERG)	1	1	
<i>Michigan</i>			
Barry (BARY)	2	2	
Kalamazoo (KMZO)	1	1	
Kalkaska (KSKA)	2	2	
Mackinac Island (MKNC)	1	1	
Oakland (OKLD)	2	2	
<i>Missouri</i>			
Pershing State Park (TERG)	1	1	
Squaw Creek NWR (TERG)	2	2	
<i>New York</i>			
Bergen (BERG)	3	3	
Cicero (CCRO)	3	3	
<i>Ohio</i>			
Grand River Lowlands Site 1 (GRL-1)	3	3	
Grand River Lowlands Site 2 (GRL-2)	3	3	
Grand River Lowlands Site 3 (GRL-3)	3	3	
Killdeer Plains (KLDR)	13	3	13
Prairie Road Fen (PRDF)	3	3	
Spring Valley (SPVY)	3	3	
Willard (WLRD)	3	3	
Wright Patterson AFB (WTPT)	2	2	
<i>Ontario</i>			
Beausoleil Island (BEAU)	3	3	
Bruce Peninsula (BPNP)	3	3	
Killbear Provincial Park (KBPP)	3	3	
Ojibway (OJIB)	2	2	
Wainfleet Bog (WAIN)	3	3	
<i>Pennsylvania</i>			
State Game Lands 95 (GLAD)	3	3	
Jennings (JENN)	3	3	
Venango (VNGO)	3	3	
<i>Wisconsin</i>			
Necedah (NCDH)	1	1	
Trempeau (TREM)	2	2	

Total sample sizes from each location, along with sample sizes for the clustering and fastsimcoal analyses, are included. Data were also generated for one additional *S. miliaris* sample that served as an outgroup to generate an unfolded site frequency spectrum for fastsimcoal analyses.

identified in the clustering analyses (see Results below), and used a script available in AftRAD to generate a SNAPP data set that consisted of unlinked biallelic SNPs for these samples. We repeated this 10 times to capture any variation associated with subsampling individuals, and performed SNAPP runs on each of the 10 data sets. The sister species *S. tergeminus* was included as an outgroup in all runs to root the trees. For all subsampled data sets, we generated maximum clade credibility consensus trees with TreeAnnotator (<http://beast.bio.ed.ac.uk/treeannotator>), and from these we obtained clade support values and 95% highest probability densities for all branch lengths separating taxa.

Mutation rates u and v were sampled as part of the analysis, and default priors as set in Beati 2 were used for the remaining run parameters. All SNAPP runs were carried out for a minimum of 5×10^6 Markov chain Monte Carlo iterations, with the first 1×10^6 discarded as burn-in, and samples collected every 1000 trees. Convergence of runs was evaluated with Tracer (Drummond and Rambaut, 2007; <http://tree.bio.ed.ac.uk/software/tracer/>), and only runs for which all ESS values exceeded 100 and no trends were observed in traces for individual parameters were retained for analyses.

In addition to the SNAPP runs described above, we performed a species delimitation analysis as implemented in BFD* (Leaché *et al.*, 2014) to further test for phylogenetic differentiation among genetic clusters. Because BFD* relies on SNAPP, this analysis was subject to similar computational demands as those described above. Therefore, we again chose three individuals to represent each group identified in our clustering analyses (*S. tergeminus*, Iowa, West and East—see clustering results below), and also included three samples to represent the Central group proposed by Ray *et al.* (2013) based on previous mtDNA analyses (these ‘Central’ samples were included as part of the Eastern group in the current analyses). These samples were used to test alternative models that ranged from a fully differentiated five-lineage model to a two-lineage model in which *S. tergeminus* and *S. catenatus* were included as the only two recognized taxa. Five independent runs of BFD* were performed, each with a different subset of individuals to represent each lineage. For each run, models were ranked based on their marginal likelihood estimates, and Bayes Factors were calculated and interpreted in the context of the recommendations provided by Kass and Raftery (1995).

Demographic model testing

Our analyses (see below) detected a phylogenetically distinct lineage within *S. catenatus* (from Iowa, USA) that, based on clustering analyses, demonstrated a pattern of genetic admixture with *S. tergeminus*. To evaluate the historical processes that may have given rise to the pattern of admixture, we tested a set of models (Figure 2) that differed based on the occurrence and timing of hybridization between *S. tergeminus* and the *S. catenatus* lineage represented by our samples from Iowa. The three models include: (1) a model in which the Iowa lineage was formed because of isolation in allopatry (‘Isolation’, Figure 2a); (2) a model in which the Iowa lineage initially diverges in allopatry, with a subsequent hybridization event between this lineage and *S. tergeminus* (‘Isolation+Hybridization’, Figure 2b); and (3) a model in which hybridization currently occurs between the Iowa lineage and *S. tergeminus*, and continues back in time for some duration that is bounded by the divergence time of the Iowa lineage (‘Ongoing Hybridization’, Figure 2c). For all models, we used the population from Killdeer Plains, Ohio, as representative of *S. catenatus* east of the Mississippi River. This population represents one of the largest and most stable populations of *S. catenatus* (Chiucchi and Gibbs, 2010) and therefore likely better represents the genetic diversity characteristic of this group than would smaller populations more strongly affected by genetic drift. Each model is described in more detail in the Supplementary Information.

A multidimensional, unfolded SFS was generated with AftRAD for model testing and parameter estimation, and included three groups: *S. tergeminus* from Ellsworth County, Kansas ($N=4$), *S. catenatus* from Buhr Access, Iowa ($N=13$) and *S. catenatus* from Killdeer Plains, Ohio ($N=13$). A maximum of one SNP was included in the SFS from each locus (unlinked-1 flag in AftRAD). We used the coalescent-based modeling package fastsimcoal version 2.5.2 (Excoffier *et al.*, 2013) to evaluate each model based on this observed SFS. fastsimcoal offers advantages over methods such as approximate Bayesian computation (Beaumont *et al.*, 2002) by efficiently generating likelihoods for genomic data sets, and relying on the SFS, eliminating the need to choose specific summary statistics upon which to base inferences. For each model, we performed 100 independent runs of fastsimcoal (30 expectation conditional maximization cycles and 5×10^4 simulations per run), and the run with the highest likelihood for each model was chosen to perform model selection with Akaike information criterion. Maximum likelihood estimates for parameters such as ancestral and current effective population sizes, times of historical events and levels of gene flow were obtained from the optimal model/run and used to generate confidence intervals by parametric bootstrapping. Specifically, we simulated 100 independent SFSs for the optimal model and parameter

Figure 2 Representation of the three models (Isolation, Isolation+Hybridization, and Ongoing Hybridization, a–c respectively) compared in fastsimcoal to evaluate the historical process giving rise to the pattern of admixture observed between *S. catenatus* samples from Iowa and the sister taxon *S. tergeminus*. These models differ in the occurrence and/or timing of hybridization between these lineages. Nearly all of the model weight from Akaike information criterion (AIC) analysis was attributed to the Isolation+Hybridization model (b).

estimates, treated each of these 100 data sets as observed data and performed parameter estimation for each in a similar way as above (50 runs, 30 expectation conditional maximization cycles and 5×10^4 simulations per run). We identified the maximum likelihood parameter estimates for each of the 100 simulated data sets, and we report the confidence intervals as the minimum and maximum values of these sets of parameter estimates. We are not aware of any direct estimates for genome-wide mutation rates in snakes and hence we used a rate of 2.5×10^{-8} , as estimated for humans (Nachman and Crowell, 2000). Finally, we evaluated how well the chosen model explains the data with a G-test statistic as used in Excoffier et al. (2013).

We followed the approach of Lande et al. (2003) to convert estimates of time from generations to years. Specifically, we assumed an age of first maturity of 3 years (Siegel, 1986) and an annual adult survival rate of 0.67 (Jones et al., 2012), and estimated a generation time of 5.03 years using the equation $T = \alpha / [s / (1 - s)]$, where T is the generation time, α is the age at first maturity and s is the annual adult survival rate.

RESULTS

Sequencing and de novo SNP identification

For the AftRAD run associated with the clustering and phylogenetic analyses, a mean of 8.2×10^5 sequence reads were assigned to each of the 91 samples (range 122 001–1 756 507) after quality filtering. The mean read depth per locus was 107.6 reads, and the median read depth was 65. A total of 19 827 nonparalogous loci were identified for the data set. Of these loci, 16 804 were monomorphic, with the remaining 3023 containing at least one polymorphic site. Of the 3023 polymorphic loci, 1466 (48.5%) were scored in at least 95% of the samples in the data set. When only *S. catenatus* was included (no *S. tergeminus* samples), a total of 19 141 loci were identified, with 17 068 of these monomorphic, 2073 polymorphic and 1019 (49.2%) of the polymorphic loci scored in at least 95% of the samples. The decreased number of polymorphic loci after *S. tergeminus* was removed is expected, as many loci that are monomorphic in *S. catenatus* samples alone become polymorphic when *S. tergeminus* is included. For the second AftRAD run in which data were generated for the fastsimcoal analyses, a mean of 6.6×10^5 reads were assigned to each of the 31 samples (range 103 469–1 596 353). The mean read depth was 85.5, and the median was 52. A total of 22 829 loci were identified. Of these, 3281 were polymorphic and 1099 were polymorphic and scored in all samples. This set of 1099 loci was used to construct the SFS for analyses in fastsimcoal.

Clustering analyses

When only *S. catenatus* samples were analyzed, Structure identified $K=3$ as optimal (maximum ΔK), with groups generally consisting of samples from (1) Iowa, (2) Illinois and Wisconsin and (3) all remaining

samples to the north and east (Figure 3a). Some moderate levels of genetic variation were shared between the Wisconsin and Illinois samples from the second group and samples from Michigan in the third group. When *S. tergeminus* samples were included in the data set, Structure identified $K=2$ as optimal, with the major division being between *S. catenatus* and *S. tergeminus*, as expected. However, *S. catenatus* samples from Iowa showed a pattern of admixture at $K=2$, in which they are assigned with $\sim 20\%$ probability (confidence interval (CI) 17.2–26.0%) to *S. tergeminus*, suggesting possible hybridization between these groups (Figure 3b).

Comparisons of Bayesian information criterion values in adegenet showed that three and four clusters were optimal for the ingroup only data set and the data set including *S. tergeminus*, respectively (Figures 4a and b). Samples in the adegenet analyses were assigned to clusters in a similar way as in the Structure analyses: *S. catenatus* individuals were separated into clusters of samples from (1) Iowa, (2) Illinois and Wisconsin and (3) all remaining samples to the north and east. All individual samples were assigned with $>99\%$ probability to these respective clusters, and when plotted along the first two discriminant function axes, the greatest differentiation between *S. catenatus* clusters was associated with the Iowa populations (Figure 4).

Finally, we used F_{st} values to estimate the magnitude of genetic divergence between the identified clusters of samples. Within *S. catenatus*, levels of divergence were lowest between the Illinois+Wisconsin group and the cluster consisting of the remaining samples to the north and east ($F_{st}=0.13$). These F_{st} values were ~ 2 –3 times higher when the Iowa cluster was compared with either of the other two *S. catenatus* clusters (0.29 and 0.32, for the two other groups, respectively). All these values were substantially less than the F_{st} observed between samples of *S. catenatus* and *S. tergeminus* (0.65).

SNAPP phylogenetic analyses

As expected from Kubatko et al. (2011), ingroup *S. catenatus* samples formed a monophyletic clade, clustering together to the exclusion of *S. tergeminus*, with high support (1.0 posterior probability) in all SNAPP runs (Figure 5). Within *S. catenatus*, Iowa samples formed a phylogenetically distinct lineage basal to the remaining ingroup samples with high support (1.0 posterior probability) in all experimental runs. In addition, the 95% highest probability density interval for branch lengths separating the Iowa group from the remaining *S. catenatus* did not include zero for any of the 10 subsampled runs (Figure 5). In contrast, the analyses did not provide support for differentiation between the Illinois+Wisconsin cluster and the cluster consisting of the remaining samples to the north and east, as

Figure 3 Results from clustering analyses in Structure with *S. catenatus* samples only (a), and with the outgroup *S. tergeminus* included (b). Each plot represents individual assignment proportions at the optimal ΔK value ($K=3$ for (a) and $K=2$ for (b)). When *S. tergeminus* is included, *S. catenatus* samples from Iowa demonstrate a pattern of genetic admixture between the two groups (b).

the highest probability density intervals for branch length between these groups included zero as a lower bound. Together, these results suggest that Iowa lineage represents a distinct phylogenetic lineage within *S. catenatus*, but that there is not strong support for the presence of phylogenetically distinct lineages among *S. catenatus* populations east of the Mississippi River.

BFD* phylogenetic analyses

Results from the independent runs of BFD* varied in terms of the specific delimitation model that was best supported. However, across all runs the best supported model was one in which samples from the Iowa group represented a distinct lineage compared with models in which Iowa samples were combined with other groups (Supplementary Table S1).

Demographic modeling

Among the demographic models in Figure 2, most of the model weight from Akaike information criterion was associated with the Isolation +Hybridization model (Table 2). This suggests that differentiation of the Iowa lineage from other *S. catenatus* involved a combination of divergence through isolation and a hybridization event that resulted in introgression between *S. tergeminus* and the Iowa lineage. Based on the values we used for the average mutation rate and generation time

in this system, maximum likelihood parameters under this model (Table 3) estimate that divergence of the Iowa lineage from other *S. catenatus* (T_{CAT}) occurred 6756 generations bp, corresponding to $\sim 34\,000$ ybp (CI 19 401–46 739 ybp), and the introgression event occurred 2242 generations bp, corresponding to $\sim 11\,000$ ybp (CI 2429–29 602 ybp). The difference in these values represents the amount of time that the Iowa lineage evolved in allopatry before the hybridization/introgression event, and is estimated to be 4514 generations or $\sim 23\,000$ years before present (CI 4502–36 095 years). A G -test to evaluate the model's fit to the data (Excoffier *et al.*, 2013) was significant ($P=0.01$), suggesting that the specified models do not provide a complete explanation of the processes generating the observed patterns of genetic variation.

The modeling analyses generated additional population parameter estimates of conservation interest (Table 3). In particular, current effective population sizes were smaller in both *S. catenatus* populations than in the *S. tergeminus* (a nonthreatened species) population: Specifically, for *S. catenatus*, the genetically effective population size (N_e) for the Killdeer Plains population was estimated at 4987 (CI 2696–9127) and the Iowa population was 7590 (CI 4344–9610), whereas the *S. tergeminus* was much larger at 90 988 (CI 54 764–96 718). In terms of historical changes in population sizes, the *S. tergeminus* population was best modeled as a growing population, whereas the two *S. catenatus* populations were best

Figure 4 Results from clustering analyses in adegenet with *S. catenatus* samples only (a), and with the outgroup *S. tergeminus* included (b). Main plots show results of discriminant analysis of principal components for the optimal *K* value inferred from Bayesian information criterion (BIC; top left insets). Within *S. catenatus*, the greatest separation in discriminant function space was observed between the Iowa samples and all the remaining *S. catenatus* samples from east of the Mississippi River.

modeled as declining populations since their divergence from each other (Table 3).

DISCUSSION

Our main results are that (1) phylogeographic analyses identify *S. catenatus* in Iowa as phylogenetically distinct from all other populations, whereas there is equivocal evidence for significant phylogenetically distinct lineages among remaining *S. catenatus* populations east of the Mississippi River; and (2) historical demographic modeling suggests that isolation and hybridization between *S. tergeminus* and Iowa *S. catenatus* has contributed to the genetic distinctiveness of Iowa snakes. We discuss the evolutionary and conservation implications of these results below.

Identifying significant phylogenetic lineages

Our results suggest that individuals from three populations located within 30 km of each other in northeastern Iowa may represent a phylogenetically distinct lineage within *S. catenatus*. This conclusion is based on a two-step analytical approach for identifying distinct lineages in a taxon that has been used in some recent species delimitation studies (that is, Leaché and Fujita, 2010; Satler *et al.*, 2013; Hedin *et al.*, 2015). The first step (discovery) involves assigning samples to putative lineages, and is followed by a second (validation) step in which the putative lineages are tested for phylogenetic distinctiveness.

For the first (discovery) step in the analysis, we used Structure (Pritchard *et al.*, 2000) and adegenet (Jombart and Ahmed, 2011) to identify genetic clusters in *S. catenatus*. These two programs differ in their approach to clustering and in the assumptions made about the data. Using the two separate approaches allowed us to evaluate the sensitivity of the genetic clusters identified to alternative clustering algorithms. Both clustering methods grouped similar sets of geographically related samples together, suggesting that the identified clusters are robust to the clustering algorithm used. However, because clusters are inferred without reference to the history of population diversification,

Figure 5 Tree summarizing results from the 10 SNAPP runs that each included different subsets of samples. Values at nodes represent posterior support for clades after the first 20% of trees were removed as burn-in (topologies and clade support values were the same across all 10 runs). Values on the branches represent the minimum and maximum values observed in the set of 95% highest probability density (HPD) values for branch length across the 10 runs. Lower bounds of the branch lengths separating Iowa from the remaining *S. catenatus* do not include zero, supporting the presence of significant phylogenetic divergence between Iowa and other *S. catenatus* populations.

Table 2 Akaike information criterion (AIC) model selection results for fastsimcoal analyses

Model	No. of parameters	Ln likelihood	AIC	Akaike weight
Isolation+Hybridization	9	−6000.05	27 649.25	1
Ongoing Hybridization	10	−6014.22	27 716.51	2.49e−15
Isolation	7	−6015.61	27 716.89	2.05e−15

Three historical demographic models differing in the presence and/or timing of hybridization between *S. tergeminus* and the Iowa lineage of *S. catenatus* were compared, and are depicted in Figure 2. Models are ordered based on relative model weights.

Table 3 Maximum likelihood estimates for demographic parameters estimated in the fastsimcoal analysis for the best-supported Isolation +Hybridization model (Figure 2b)

Parameter	Point estimate	Confidence interval
T_{SIS}	30 894 (155 397)	24 287–34 721 (122 164–174 647)
T_{CAT}	6756 (33 988)	4953–9289 (24 914–46 723)
T_{HYB}	2242 (11 277)	687–4781 (3456–24 048)
P_{TERG}	0.066	0.021–0.17
N_{SIS}	34 142	26 285–40 824
$N_{\text{CAT_ANCES}}$	32 730	20 359–44 632
N_{TERG}	90 988	61 200–95 533
N_{IOWA}	7590	4344–9610
N_{KCLR}	4987	3911–9127

Times are given in number of generations, with the number of years included in parentheses based on the estimated generation time. Confidence intervals for parameters were generated from parametric bootstrapping, as described in the Materials and methods. Parameters are described in Supplementary Information.

they may not directly reflect phylogenetic branching patterns (Kalinowski, 2011). Therefore, the second (validation) step in our approach utilized the species tree-based programs SNAPP and BFD* to evaluate the phylogenetic distinctiveness of each identified cluster. Results from these phylogenetic analyses strongly support Iowa as a distinct lineage, but provide equivocal support for significant phylogenetic differentiation between genetic clusters east of the Mississippi River, with some analyses supporting distinctiveness whereas others do not. We conclude that these clusters east of the Mississippi River have not been isolated for a sufficient time following the post-glacial colonization of their present range to show evidence of being evolutionarily independent lineages.

These conclusions differ from those of Ray *et al.* (2013) in terms of the number and geographic location of potential conservation units within *S. catenatus*. That study was limited to a single mtDNA locus, whereas our analyses used > 1000 nuclear DNA loci. Although mtDNA alone can reveal phylogeographic patterns that are consistent with genome-wide patterns (Barrowclough and Zink, 2009), analyses based on multiple nuclear DNA markers often provide a more reliable estimate of the true phylogeographic history of a species (Edwards and Bensch, 2009). In addition, Ray *et al.* (2013) used a clade support metric (the *gsi* statistic; Cummings *et al.*, 2008) to define conservation units that is less stringent than our more conservative approach in which we assessed and required differentiation across a series of metrics including clade support values, branch lengths and BFD* analyses.

Hey (2009) discussed the arbitrary nature of delineating formal biological groupings such as species or conservation units, and we acknowledge that judgment of what constitutes distinct phylogenetic lineages in our study depends on the specific criteria applied. As shown by Hedin *et al.* (2015), groups characterized by strong population structure present some of the most challenging systems for

delimitation, especially when inferences are limited to genetic data. In such cases, methods for delineating formal groups (that is, species, conservation units) may ‘over-split’ major genetic groups, and factors such as sample size can play an important role in the inference of whether such groups are recognized or not (see Niemiller *et al.*, 2012 for an empirical example). As demonstrated by Chiucchi and Gibbs (2010), *S. catenatus* exhibits characteristics such as strong population structure and small population sizes that may make these snakes susceptible to potential ‘over-splitting’ in lineage delimitation studies. Factors such as these led Carstens *et al.* (2013a) to highlight the importance of using conservative criteria for delimiting evolutionary groups, and Hedrick (2001) has also emphasized the need to be conservative when using neutral genetic markers to identify conservation units such as evolutionary significant units. Given these points, we emphasize that our main result, that the greatest degree of genetic divergence within *S. catenatus* occurs between lineages east and west of the Mississippi River, is not affected by the specific choice of criteria. However, even this ‘large’ divergence within *S. catenatus* is relatively small (that is, branch lengths and F_{st} values are considerably smaller) compared with the divergence between *S. catenatus* and its sister taxon *S. tergeminus*. For these reasons, we suggest that the differentiation observed between the Iowa groups and the remaining *S. catenatus* reflects intraspecific rather than interspecific diversity.

Previous analyses failed to detect Iowa populations of *S. catenatus* as distinct. We see several potential reasons for this. Most simply, samples from Iowa were not included in Kubatko *et al.* (2011), and hence their distinctness at the SNP-based nuclear DNA loci used in that study could not be evaluated. Ray *et al.* (2013) included five samples from the Iowa populations surveyed here (three of these five samples were included in the present study) and found that these samples fell within their Western *S. catenatus* mtDNA clade that also included samples from Wisconsin and Illinois. Given our conclusion that past hybridization occurred between Iowa snakes and *S. tergeminus*, the fact that no *S. tergeminus* mtDNA was observed in these samples could be explained in a number of ways. These include loss of rare *S. tergeminus* mitochondrial genomes because of drift, sampling effects or the possibility that introgression was primarily a result of successful hybridization between male *S. tergeminus* and female *S. catenatus* (see Toews and Brelsford, 2012 for a review on discordant patterns between mtDNA and nuclear DNA). At a minimum, the mtDNA results from Ray *et al.* (2013) demonstrate that the Iowa lineage has not been isolated for a sufficient period to achieve monophyly at the sampled mtDNA locus, constituting one criterion previously proposed and applied for designating evolutionary significant units for conservation purposes (Moritz, 1994). Regardless, our study demonstrates the value in combining genomic-scale data sets with broad geographic sampling for identifying potentially important cryptic genetic diversity within taxa.

Origin of the Iowa lineage

Historical demographic modeling has been used to identify the evolutionary mechanisms responsible for observed patterns of lineage diversity within species (Carstens *et al.*, 2013b). Analysis using Structure showed a pattern consistent with genetic admixture in the Iowa snakes that could reflect the effects of hybridization, incomplete lineage sorting or both. Our modeling approach incorporated in the program fastsimcoal provided strong support for a two-step process in which the lineage represented by the Iowa samples initially diverged in allopatry, and then became genetically admixed thousands of years later because of introgression with *S. tergeminus*. Thus, the distinctiveness of the Iowa snakes is because of the joint effects of divergence

in allopatry before and after a hybridization event, and the introgression of novel genetic material via a historically limited hybridization event.

In terms of isolation, two mechanisms could contribute to the initial divergence between *S. catenatus* samples from Iowa and the remaining populations to the east: refugial isolation or large-scale vicariance. One possibility is that the group from Iowa represents a refugial population from the 'Driftless Area' that remained unglaciated during the Wisconsin glacialiation (Holliday *et al.*, 2002). Phylogeographic structure associated with the Driftless Area has been documented in a number of taxa (Rowe *et al.*, 2004; Lee-Yaw *et al.*, 2008). However, our Wisconsin samples are also likely from the Driftless Area, and yet do not show a similar pattern of divergence as the Iowa samples, as expected under this hypothesis.

Vicariance effects due to the influence of the Mississippi River as a long-term isolating barrier could also potentially explain the differentiation. Similar patterns of divergence coinciding with the Mississippi River have been demonstrated in other snakes including *Diadophis* spp. (Fontanella *et al.*, 2008) and *Lampropeltis* spp. (Pyron and Burbrink, 2009), supporting this hypothesis. To our knowledge, the known distribution of *S. catenatus* populations west of the Mississippi River is currently restricted to the area represented by our samples. Additional sampling is needed to confirm the restricted range of this lineage. If populations were identified from other geographic areas, these samples could be used to further test vicariance because of the Mississippi River as a diversifying mechanism that could have potentially influenced other *S. catenatus* populations on the western extreme of the species distribution.

Hybridization has been previously reported between related species of rattlesnakes (Murphy and Crabtree, 1988) including these species of *Sistrurus* (Evans and Gloyd, 1948, but see Gibbs *et al.*, 2011). Our modeling approach allowed us to estimate parameters related to historical demographic events such as the timing of divergences and admixture events. We note that these estimates are dependent on a number of factors that include the assumed mutation rate for our markers and generation times for these snakes, the appropriateness of the model under which the values were estimated and the degree to which any linkage among markers may not have been accounted for. For such reasons specific values should be interpreted with caution. With this in mind, our parameter estimates date the divergence of the Iowa lineage at ~34 000 ybp and the time at which introgression occurred at ~11 000 ybp. These dates roughly correspond with the late Pleistocene and early Holocene eras, respectively. This time was characterized by retreat of the Wisconsin glacial ice sheet, altered habitats and associated distribution shifts for many species (Schmidt, 1938; King, 1981; Webb, 1981) that could have led to secondary contact between the Iowa lineage and *S. tergeminus*. These dates suggest that introgression occurred on the order of 1000s of years before any significant human impacts on the landscape that in this region of North America resulted from modern agriculture and development in the nineteenth and twentieth centuries. Thus, the introgression appears to have occurred as a natural process, as opposed to being caused by human activities, and hence the observed pattern of admixture is not likely to represent recent genetic swamping such as has been observed in other threatened taxa (Allendorf *et al.*, 2001). In contrast, human impacts on habitat may have had the opposite impact: habitat conversion to agriculture and resulting fragmentation now severely limit the distributions of *Sistrurus* populations in Iowa, and there are currently no sympatric populations of *S. tergeminus* and *S. catenatus* in the state (VanDeWalle and Christiansen, 2002), eliminating the potential for any gene exchange on contemporary timescales.

Conservation status of Iowa snakes

A final question is whether the genetically distinct Iowa snakes warrant special conservation status. Our results suggest that Iowa snakes diverged from other *S. catenatus* as a result of both isolation and historical hybridization over evolutionary timescales. Isolation can help promote local adaptation through selection acting in different environments, and this is the primary mechanism suggested to be responsible for adaptive differences between evolutionary significant units (Crandall *et al.*, 2000). In terms of hybridization, we conclude that this lineage represents an example of either a Type 1 (Natural Hybrid Taxon) or Type 2 (Natural Hybridization) scenario under the scheme of Allendorf *et al.* (2001) for characterizing hybridization in threatened species. Allendorf *et al.* (2001) argue that rare hybrid groups originating through these scenarios should be considered for protection because hybridization can be an important evolutionary process capable of creating novel genetic diversity. The fact that Iowa snakes also appear to be stabilized, introgressed individuals that have persisted over evolutionary timescales suggests that they may contain a unique set of adaptive variation within the species as a whole. However, the presence or absence of adaptive variation cannot be inferred given the (presumably) neutral markers used in this study. Additional studies of divergence at functional loci (Funk *et al.*, 2012), phenotypic comparisons of Iowa snakes with other *S. catenatus* populations or ecological studies including Environmental Niche Modeling (Wooten and Gibbs, 2012) are needed to establish whether Iowa snakes contain unique adaptive variation, as little is currently known in this regard. Until this work is completed, we feel our analyses suggest that a conservative conclusion is that Iowa snakes represent a distinct portion of the evolutionary history of *S. catenatus* as a whole, and hence deserve consideration as a Distinct Population Segment under the US Endangered Species Act because of their discreteness, significance and population status within the species. We emphasize that additional considerations independent of genetics such as local abundance and ecological importance are also important in establishing the most appropriate conservation status of these snakes.

DATA ARCHIVING

RADseq data sets are available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.nt65q>. Historical demographic models used in the Fastsimcoal analyses are available at <https://github.com/mikesovic>.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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