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Phylogenetic, population genetic, and morphological analyses reveal evidence for

one species of Eastern Indigo Snake (*Drymarchon couperi*)

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Abstract

Accurate species delimitation and description are necessary to guide effective conservation management of imperiled species. The Eastern Indigo Snake (*Drymarchon couperi*) is a large species in North America that is federally-protected as Threatened under the Endangered Species Act. Recently, two associated studies hypothesized that *Drymarchon couperi* is two species. Here, we use diverse approaches to test the two-species hypothesis for *D. couperi*. Our analyses reveal that (1) phylogenetic reconstruction in previous studies was based entirely on variance of mitochondrial DNA sequence data, (2) microsatellite data demonstrate significant population admixture and nuclear gene flow between mitochondrial lineages, and (3) morphological analyses recover a single diagnosable species. Our results are inconsistent with the two-species hypothesis, thus we reject it and formally place *Drymarchon kolpobasileus* into synonymy with *D. couperi*. We suggest inconsistent patterns between mitochondrial and nuclear DNA may be driven by high dispersal of males relative to females. We caution against species delimitation exercises when one or few loci are used without evaluation of contemporary gene flow, particularly species with strong sex-biased dispersal (e.g., squamates) and/or when results have implications for ongoing conservation efforts.

Introduction

Accurate species delimitation and description are critical not only for understanding global patterns of biodiversity, but also to guide effective conservation strategies [1–4]. For example, species are often delimited into multiple species on the basis of systematic studies

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51   utilizing molecular genetic data, thereby requiring adjustment of existing conservation

52   management plans (e.g., [5]). When species delimitation methods fail to correctly diagnose

53   individuals (*sensu* [6]), such errors can have significant consequences for conservation and

54   management of imperiled species by reducing or diverting finite conservation resources [2].

55   Therefore, taxonomic division into multiple species should be performed carefully and only

56   when robust evidence supports a decision to revise. Indeed, authors have cautioned that studies

57   of species delimitation should be conservative, because "it is better to fail to delimit species than

58   it is to falsely delimit entities that do not represent actual evolutionary lineages" [7].

59   The Eastern Indigo Snake (*Drymarchon couperi*) is a large colubrid native to the Coastal

60   Plain of the southeastern United States. However, *D. couperi* populations have declined in

61   abundance precipitously over the last century, largely due to habitat loss, habitat fragmentation,

62   and historical over-collecting for the pet trade [8,9]. As a result of these declines, *D. couperi* is

63   listed as Threatened under the U.S. Endangered Species Act [9,10]. Potentially viable

64   populations of *D. couperi* remain in large contiguous habitats in southeastern Georgia [11–13],

65   and throughout peninsular Florida [13,14], but the species was likely extirpated from

66   Mississippi, Alabama, and the Florida panhandle [13].

67   Current conservation management plans for *D. couperi* were developed under the

68   hypothesis that *D. couperi* represents a single species. However, this hypothesis was recently

69   challenged by Krysko et al. [15], who used DNA sequence analyses to describe two genetic

70   lineages of *D. couperi* – an Atlantic lineage, including populations in southeastern Georgia and

71   eastern peninsular Florida, and a Gulf lineage of populations in western and southern peninsular

72   Florida and the Florida panhandle. This phylogeographic study was followed by a second paper

73   [16] that analyzed morphological variation between the Atlantic and Gulf lineages and provided

74   an official description of the Gulf lineage as a purported novel species, the Gulf Coast Indigo

75   Snake (*Drymarchon kolpobasileus*).

76   Given the conservation status of *D. couperi* (*sensu lato*), these results have potentially

77   important consequences for the conservation of Eastern Indigo Snakes. First, division of *D.*

78   *couperi* (*sensu lato*) into two smaller-ranged species results in two species with substantially

79   smaller population sizes that are, therefore, at greater risk of extinction (*sensu* [2]; e.g., [17]).

80   Second, conservation and recovery of two rare species requires more time and funds than one,

81   and both resources are in short supply. Finally, as noted by Krysko et al. [15], active

82   conservation management plans for *D. couperi* (*sensu lato*) include population repatriation

83   projects in Alabama and the Florida panhandle, where populations attributed to the Gulf lineage

84   presumably were extirpated. Repatriation projects should be informed by phylogeographic and

85   genetic developments [18]. The description of *D. kolpobasileus*, therefore, causes increased

86   logistical complexity for Eastern Indigo Snake captive breeding and repatriation projects.

87   We represent additional experts on Eastern Indigo Snake taxonomy, ecology and

88   conservation, many of whom participated in an inter-agency workshop on Eastern Indigo Snake

89   taxonomy referenced in Krysko et al. [15,16]. During that event, consequences of the discovery

90   that Eastern Indigo Snakes comprise two genetic lineages were debated, and this debate was used

91   to inform conservation plans for the species. However, skepticism was voiced that the two

92   lineages represent distinct evolutionary species, based largely on description of microsatellite

93   data documenting widespread admixture of the lineages. Here, we formally address that debate

94   to evaluate the hypothesis that *D. couperi* comprises two species [15,16]. We adopted a unified

95   species concept [19,20] to define and operationally diagnose species. Under this concept, species

96   are necessarily defined as independently evolving metapopulation lineages and this feature must

97   necessarily be demonstrated to delimit species [20]. However, we also evaluated additional lines

98   of evidence, such as morphological diagnosability, to provide secondary assessments of lineage

99   separation. Together, we sought to provide a conceptually robust and integrative test [21,22] of

100   whether *D. couperi* is two distinct species.

101   In this paper, we first re-analyze gene sequence data presented by Krysko et al. [15] to

102   infer whether their phylogeny was overly-influenced by data from the mitochondrial genome.

103   Second, we analyze a novel microsatellite DNA dataset and test for evidence of population

104   admixture and contemporary gene flow between the two genetic lineages identified by Krysko et

105   al. [15] as an explicit test of whether there are two independently evolving metapopulation

106   lineages of *D. couperi*. Specifically, if *D. couperi* is two species, we predicted little or no

107   admixture between hypothetical species, particularly at the putative contact zone identified by

108   Krysko et al. [15,16]. Third, we analyze new morphological data collected from 125 Eastern

109   Indigo Snakes, including individuals from both genetic lineages of Krysko et al. [15,16].

110   Specifically, we evaluate the diagnostic features of head and scale shape presented by Krysko et

111   al. [16] as tests of whether morphological phenotypes are secondary lines of evidence supporting

112   previously delimited species. Last, we review features of the life history of *D. couperi* and

113   suggest how they inform interpretations of genetic and morphological data.

114

Materials and Methods

Gene sequence analysis

117   To infer evolutionary history among populations of *D. couperi* (*sensu lato*), Krysko *et al.*

118   [15] analyzed sequence data obtained from three genetic markers: the linked mitochondrial

119   (mtDNA) genes cytochrome *b* (CytB) and nicotinamide adenine dinucleotide dehydrogenase

120   subunit 4 (ND4) and the nuclear gene *neurotrophin*-3 (NT3). The authors estimated phylogenetic

121   relationships among populations by analyzing a concatenated dataset including both

122   mitochondrial and nuclear loci. These data were evaluated with maximum likelihood (ML) and

123   Bayesian analyses; for the Bayesian analysis, the dataset was partitioned such that nucleotide

124   substitution was modeled separately for each locus. Because both analyses generated similar

125   phylogenetic hypotheses, the authors described results only from the Bayesian analysis.

126   Phylogenetic analyses of a single or few genetic loci frequently describe evolutionary

127   patterns that do not reflect the organism's true evolutionary history (i.e., the gene tree/species

128   tree problem; [1,23–25]). In particular, use of and reliance on mtDNA for phylogenetic and

129   taxonomic analyses has been criticized because mtDNA has a vastly different natural history

130   than the primary mode of genetic inheritance, nuclear DNA (nDNA). Mitochondrial DNA has a

131   lower effective population size, higher mutation rate, and frequently defies critical assumptions

132   of neutral evolution by being under selection [4,26]. More importantly, mtDNA is maternally

133   inherited and, therefore, may not describe an organism's true patterns of inheritance expressed

134   through the nuclear genome [26]. This is particularly problematic for species with relatively low

135   dispersal rates that are more likely to show phylogeographic breaks that are not driven by

136   decreased gene flow but by chance alone [27], or for species with intersexual differences in

137   movement, site fidelity, or breeding behavior [28–30].

138   Given these and other limitations, a customary practice in phylogenetic studies is to use

139   both mitochondrial and nuclear loci and to describe phylogenetic patterns inferred from these

140   two components of the genome separately (e.g., [24,31–33]). This practice can help identify

141   situations for which phylogenetic hypotheses generated from mtDNA (1) are incongruent with

142   hypotheses from the nuclear genome and that (2) might be erroneously assumed to accurately

143   depict the species tree. However, Krysko *et al.* [15] combined the mitochondrial and nuclear

144   markers and used that concatenated dataset to infer both ML and Bayesian phylogenies from the

145   combined datasets. We therefore argue that the DNA sequence analyses of Krysko et al. [15] are

146   biased toward describing patterns from maternally inherited mtDNA and require re-evaluation.

147   To explore the extent to which nuclear sequence data support lineage divergence and

148   speciation within Eastern Indigo Snakes, we accessed the Krysko *et al.* [15] sequence data from

149   GenBank (S1 Table) and used ML methods to infer a nuclear gene tree from the NT3 dataset,

150   following the methods used by Krysko *et al.* [15]. This dataset included 23 *D. couperi* (*sensu*

151   *lato*) samples (N = 13 Atlantic clade, N = 10 Gulf clade) and four outgroup taxa (*Drymarchon*

152   *melanurus erebennus*, *Drymarchon melanurus rubidus*, *Coluber constrictor*, *Masticophis*

153   *flagellum*). We fit different models of nucleotide substitution and ranked them using BIC; this

154   procedure suggested that the Kimura [34] model best fit the data. We then estimated a ML

155   phylogeny for NT3 using the package 'phangorn' [35] in the statistical Program R [36]. This

156   method allowed us to test whether a phylogenetic pattern inferred from nuclear DNA alone was

157   consistent with or different from a concatenated analysis of mitochondrial and nuclear DNA.

158

Microsatellite analysis

160   To test whether mitochondrial lineages of *D. couperi* represent independently evolving

161   metapopulation lineages, we extracted and genotyped microsatellite DNA from 428 tissue

162   samples (scale, shed skins, or muscle from road-killed individuals) throughout peninsular Florida

163   and southern Georgia. Twenty-five samples were obtained from the collections of the Florida

164   Museum of Natural History, including 20 samples used in Krysko *et al.* [15]. The samples from

165   Krysko *et al.* [15] included individuals from central Florida that represented both mitochondrial

166   clades where they occur in close proximity. The remaining Florida samples (N = 170) were

167   collected during field studies of *D. couperi* [37,38] in and around Highlands County or

168   opportunistically by authorized project partners. The samples from Georgia (N = 233) were

169   collected by multiple project partners as part of a study of population fragmentation in the state

170   (S. Spear et al., unpublished data). Our samples include similar representation of both

171   mitochondrial lineages (55% Atlantic and 45% Gulf). We extracted DNA using the Qiagen

172   DNeasy blood and tissue extraction kit (Qiagen, Inc., Valencia, CA). We ran 17 microsatellite

173   loci [39] within three multiplexed panels using the Qiagen Multiplex PCR kit (S2 Table for

174   details). Each reaction contained 1X Qiagen Multiplex PCR Master Mix, 0.2 µM multiplexed

175   primer mix (each primer at equal concentrations), and 1 µl of DNA extract in a total volume of 7

176   µl. The PCR protocol was modified from Shamblin et al. [39] for multiplex PCR and consisted

177   of an initial denaturation of 95ºC for 15 min, 20 touchdown cycles of 94°C for 30 s, 60°C minus

178   0.5°C per cycle for 90 s and 72°C for 1 min, followed by 30 cycles of 94°C for 30 s, 50°C for 90

179   s and 72°C for 1 min, and a final elongation step of 60°C for 30 min. Multiplexed PCR products

180   were run on a 3130xl Applied Biosystems Genetic Analyzer at the University of Idaho's

181   Laboratory for Ecological, Evolutionary, and Conservation Genetics. We scored fragment sizes

182   using Genemapper 3.7 (Applied Biosystems).

183   We tested for the presence of null alleles that would lead to violations of Hardy-

184   Weinberg equilibrium assumptions using the software FreeNA [40] and excluded any loci that

185 had an estimated null allele frequency > 0.10. We estimated population structure and number of

186 genetic clusters using the Bayesian clustering algorithm Structure 2.3.4 [41]. We used the

187 admixture model with 100,000 iterations following 10,000 burn-in repetitions. We evaluated K =

188 1–10 with five replicates for each value of K. We used Structure Harvester [42] to implement the

189 Delta K method of Evanno et al. [43] to estimate the number of clusters that best explain the

190 microsatellite data. We used CLUMPP v.1.1.2. [44] to estimate the optimal cluster assignment in

191 a single file based on the five replicates for the best supported values of K. Because the Evanno

192 et al. [43] method analyzes changes in likelihood between values of K, it cannot estimate Delta K

193 for K = 1; therefore, we assessed the probability of a K = 1 scenario with the raw likelihood

194 values from Structure. Given biases of methods to estimate population structure from

195 microsatellite data [45], we sought to follow recommendations from Janes et al. [45] by

196 describing and comparing population structure predicted by both the Delta K and raw likelihood

197 outputs from Structure, while also reporting bar plot outputs for different values of K supported

198 by those methods. We used the program CLUMPAK [46] to visualize bar plot outputs from

199 Structure. We note that during these exploratory hierarchical analyses we observed additional

200 fine scale genetic structure among populations; these details were outside the scope of the current

201 analysis, but we intend to examine these data more fully in a future paper. Finally, we tested for

202 evidence of population differentiation in the 20 samples used by Krysko *et al.* [15] that

203 represented spatial overlap of Atlantic and Gulf clades by estimating Jost's D metric of genetic

204 differentiation [47]. Jost's D was developed to better represent actual levels of genetic

205 differentiation when markers with high mutation rates (such as microsatellites) are used. We

206 estimated Jost's D using the 'mmod' package [48] in R.

207   We also conducted a spatial principle components analysis (sPCA) to identify spatial

208   patterns of genetic structure while accounting for spatial autocorrelation among samples without

209   relying on assumptions of Hardy-Weinberg equilibrium [49]. sPCA requires specifying a

210   connection network to define connected samples. We evaluated three connection networks: (1)

211   Delaunay triangulation, (2) Gabriel graph, and (3) a distance-based connection network, where

212   samples ≤ 22.2 km (the maximum known dispersal distance by Eastern Indigo Snakes; [50])

213   were considered connected. We conducted significance tests for global and local structure using

214   9,999 permutations in the package 'adegenet' [51] in R.

215   Last, we analyzed the microsatellite data using linear mixed-effects models with

216   maximum-likelihood population effects (MLPE; [52]) to better understand the role of isolation

217   by distance in explaining genetic distance within and among clades. We estimated genetic

218   distance at the individual level using 1 - proportion of shared alleles [53], where increased values

219   indicated greater genetic dissimilarity between samples. To test for isolation by distance, we

220   built three MLPE models examining how genetic distance varied by Euclidean geographic

221   distance: a model using only Atlantic clade samples, a model using only Gulf clade samples, and

222   a model using all sample from both clades. For each model, we report the parameter estimate for

223   Euclidean distance, it's standard error and t statistic, and the marginal R^2 (i.e., the proportion of

224   variance explained by fixed-effect factors; [54,55]). To visualize how isolation by distance

225   differed within and between clades, we graphically overlaid genetic distance against Euclidean

226   distance for within-clade distances against genetic distances measured between clades to see how

227   isolation by distance varied within clades relative to across all samples. If mitochondrial lineages

228   represented different species, we predicted to observe lower genetic distances within clades than

229   between clades (i.e., no or little overlap in genetic distances within and among clades). We

230   estimated proportion of shared alleles using the package 'adegenet' [51] and implemented MLPE

231   tests with individuals as random effects using the package 'ResistanceGA' [56] in R.

232

Morphological analysis

234   Krysko et al. [16] performed principal components analyses (PCA) on five linear

235   measurements of head morphology: head length, head height, length of a temporal scale, length

236   of the 7th infralabial, and width of the 7th infralabial. Head length, head height, and 7th infralabial

237   length loaded positively and 7th infralabial width loaded negatively on the first principal

238   component, which explained 44.7% of variance. This axis separated specimens that were

239   described to be long- and wide-headed with long and narrow 7th infralabials (high PC1 scores;

240   almost exclusively Atlantic lineage snakes) from specimens that were described to be short and

241   narrow-headed with short and wide 7th infralabials (low PC1 scores; almost exclusively Gulf

242   lineage snakes). Temporal length loaded heavily and positively on the second principal

243   component, which explained 25.4% of variance and separated specimens with relatively elongate

244   temporals (high values on PC2; primarily Atlantic lineage) from specimens with relatively short

245   temporals (low values on PC2; primarily Gulf lineage). The authors then used these axes to

246   justify presentation of the shape of the 7th infralabial, temporal, and head as being diagnostic of

247   each species.

248   We found inconsistencies in the head scale and shape characters measured by Krysko et

249   al. [16], which caused us to question their validity as diagnostic features. In particular, the

250   specific temporal scale identified as being measured differed between Figures 3 and 5 of Krysko

251   et al. [16]. This resulted because the number and position of temporal scales within species of

252 *Drymarchon* is variable (Fig 1). Similarly, Figure 3 of Krysko et al. [16] appears to identify the

253 6th infralabial rather than the 7th as the scale measured for multivariate analysis. Thus, it is

254 unclear which scale was measured for this important character and whether the same scale was

255 measured among all individuals included in analysis.

256 Fig 1. Head scale patterns in Eastern Indigo Snakes (*Drymarchon couperi*). A) 2+2 condition of

257 temporals (I = dorsal anterior temporal; II = ventral anterior temporal; III = dorsal posterior

258 temporal; IV = ventral posterior temporal) and position of 4th, 5th, 6th, and 7th infralabials; B)

259 3$_v$+2 condition of temporals (extra ventral temporal shaded); C) 4+2 condition of temporals

260 (extra dorsal and ventral temporals shaded); D) 3$_d$+2 condition of temporals (extra dorsal

261 temporal shaded).

262 To better understand whether morphological variation provides secondary support for

263 lineage divergence and speciation of *D. couperi*, we examined 114 individuals housed at the

264 Orianne Center for Indigo Conservation, a sample that included 68 and 17 specimens from the

265 Atlantic and Gulf lineages of Krysko et al. [15], respectively, along with 4 specimens of

266 unknown lineage assignment, 12 specimens derived from F1 hybrids of Atlantic and Gulf lineage

267 snakes, and 13 hybrids of Atlantic lineage snakes crossed with adults of unknown origin. Lateral

268 or dorsolateral photos of the head were taken of each specimen, including a millimeter ruler for

269 scale. The photos were used to record the condition of the temporal scales for each specimen.

270 Four categories were recognized based on the number and position of temporal scales (Fig 1).

271 We generated a contingency table providing counts of specimens in each of the four categories

272 for each lineage, specimens of unknown lineage, and hybrids. Fisher's exact test was used to

273 determine whether the relative proportions of temporal scale categories differed between the

274 Atlantic and Gulf lineages. Additionally, we measured total head length (posterior-most point of

275   8th supralabial to anterior tip of rostral; N = 111), head height (only for photos in lateral aspect; at

276   level of anterior-most point of parietal suture; N = 35), and length of the dorsal posterior

277   temporal (intersection of ventral posterior temporal, dorsal posterior temporal, and adjacent first

278   dorsal scale to intersection of ventral posterior temporal, dorsal posterior temporal, and adjacent

279   ventral temporal; N = 111). All distances were measured using Adobe Photoshop 6.0 with

280   reference to the photographed ruler. We used an analysis of covariance (ANCOVA) to test

281   whether the linear relationship between head length and head height differed between Atlantic

282   and Gulf lineages. We divided the length of the dorsal posterior temporal by head length to

283   control for effects of body size and used an analysis of variance to test whether temporal length

284   differed among the four categories of temporal scales.

285   We also examined 11 preserved specimens in the Auburn University Museum (AUM)

286   collections. These snakes were from southeastern Georgia and presumed to belong to the

287   Atlantic lineage. For these specimens, we measured length and width of the 6th and 7th infralabial

288   scales with dial calipers. We measured both scales because it was not clear which of these was

289   measured by Krysko et al. [16] and because they represent the position of the 7th lower labial

290   scale if the mental scale was included and if it was excluded. Additionally, we used photos of the

291   type specimens presented in Krysko et al. [16] to determine length and width of the 6th and 7th

292   infralabial scales using Adobe Photoshop 6.0. A length-to-width ratio was then calculated for

293   each specimen. Mean differences between 6th and 7th infralabials were tested with a paired t-test.

294   Differences between our sample of Atlantic lineage snakes and the type specimens was

295   determined by visual inspection. We used SAS v.9.4 for all analyses (SAS Institute, Inc., 2008)

296   with $\alpha = 0.05$.

297   Ethics Statement

298 The use of live snakes for research was approved by Auburn University IACUC protocols (PRN

299 2007-1142, 2010-1750, 2013-2386, 2017-3102) and a federal research permit from the United

300 States Fish and Wildlife Service (TE32397A-O).

301

Results

Gene sequence analysis

304 We found that the nuclear locus NT3 was completely invariant across all *D. couperi*

305 specimens, and thus NT3 had no variable sites for phylogenetic inference of *D. couperi*. As

306 expected, the inferred ML phylogeny estimated a polytomy (Fig 2), indicating a lack of

307 phylogenetic structure among individuals from the two mitochondrial lineages of *D. couperi*

308 (*sensu lato*) [15]. Specimens of *D. melanurus erebennus* and *D. melanurus rubidus* clustered as

309 sister taxa to the exclusion of all *D. couperi* specimens when rooted by *Coluber constrictor* +

310 *Masticophis flagellum*.

311 Fig 2. Maximum likelihood phylogeny among samples of Eastern Indigo Snakes (*Drymarchon*

312 *couperi, sensu lato*) and outgroups (*Drymarchon melanurus*, *Coluber constrictor*, *Masticophis*

313 *flagellum*), as inferred from sequence data from the nuclear gene *neurotrophin*-3 (NT3). Indigo

314 snakes are labeled by GenBank accession numbers and are classified by the lineages identified

315 by Krysko et al. [15]. Taxon labels: blue = Atlantic lineage, orange = Gulf lineage).

316

Microsatellite analysis

318   We found evidence for null alleles in three loci (Dry33, Dry63, and Dry69). Therefore,

319   we eliminated these loci from further analysis and retained the remaining 14 loci. Delta K was

320   maximized at K = 2 (Fig 3) and supported two genetic clusters: one associated with the northern-

321   most samples, and a second with the southern-most samples. However, there was extensive

322   admixture between the two clusters, particularly in central Florida (Fig 4; Fig 5). For the 20

323   samples from Krysko et al. [15], including representatives from Atlantic and Gulf lineages, we

324   found all individuals were assigned to our southern cluster, and all were highly admixed with the

325   northern cluster (0.66 assignment to the southern cluster for the Gulf clade and 0.57 assignment

326   to southern cluster for the Atlantic clade). Furthermore, the Jost's D value for these 20 samples

327   was extremely small (0.0004), indicating no genetic differentiation among samples at the

328   putative contact zone. Raw likelihood values from Structure began to plateau at K = 3 (Fig 3),

329   and we interpreted this as support for three populations: a cluster of the southern samples, but

330   described further population subdivision of the northern samples into two clusters.

331   Fig 3. Plot of Delta K (A) and likelihood scores (B) used to identify the most likely number of

332   population clusters across the range of *Drymarchon couperi* using the Bayesian algorithm

333   Structure [43]. The dashed line in (A) indicates Delta K = 0; the error bars in (B) indicate S.D.

334   Fig 4. Bar plots of population clustering estimated through the Bayesian clustering algorithm

335   Structure with (A) K = 2 and (B) K = 3. The y-axis is the proportion of individual ancestry for

336   each cluster; in the x-axis, group 1 represents individuals assigned or assumed to be within the

337   Atlantic mitochondrial clade, and group 2 represents individuals assigned or assumed to be in the

338   Gulf mitochondrial clade. Within each group, individuals are sorted by latitude. (A) K = 2; blue

339   indicates alleles from the northern population cluster, and orange indicates alleles from the

340   southern population cluster. (B) K = 3; purple and blue indicate alleles from two northern

341   population clusters, and orange indicates alleles from the southern population cluster.

342   Fig 5. Maps of sampling sites represented as pie charts of percent ancestry within population

343   clusters identified by Structure analyses. (A) K = 2 populations, with the southern cluster

344   represented as black and the northern cluster as gray; (B) K = 3 populations, with the southern

345   cluster as black and two northern populations as grey and white. Lines indicate state boundaries

346   in the southeastern U.S.A. Pie charts for both panels are colored as in Fig 4.

347   Inferences about spatial genetic structure were very similar among the three connection

348   networks we used, so we report the results using the distance-based connection network (see SI

349   Figures S1 and S2 for results using the Delaunay triangulation network). Lagged scores from the

350   first two PC axes were highly correlated between different connection networks ($r \geq 0.93$). The

351   global test was significant (observed = 0.0222, $P < 0.0001$) while the local test was not (observed

352   = 0.0064, $P = 0.84$). The first PC axis explained the most variation followed by the second PC

353   axis (0.2978 and 0.1533, respectively; all other axes ≤ 0.1002; SI Figure S3) and both axes

354   showed positive spatial autocorrelation (Moran's $I = 0.83$ and 0.74, respectively; SI Figure S3).

355   The lagged scores of the first axis suggested genetic structure followed a north-south gradient,

356   while the second axis suggested strongest genetic structure within southern Georgia and between

357   Georgia and Florida (Fig 6). There was no consistent differentiation between samples from

358   different lineages and relatively substantial overlap across the putative contact zone.

359   Fig 6. Spatially lagged scores for each sample from the first (A) and second (B) axes of a spatial

360   principle components analysis (sPCA). Atlantic clade samples are displayed using circles while

361   Gulf clade samples are displayed using squares. Samples with more extreme values/colors are

362   more genetically differentiated.

363   MLPE models described strong effects of Euclidean distance on genetic distance among

364   samples. Specifically, we observed significant effects of Euclidean distance on genetic distance

365   within the Atlantic clade ($\beta = 0.05$, SE = 0.0007, $t = 65.5$, $R^2 = 0.21$), the Gulf clade ($\beta = 0.07$,

366   SE = 0.001, $t = 48.6$, $R^2 = 0.34$), and among all samples ($\beta = 0.04$, SE = 0.0003, $t = 148.8$, $R^2 =$

367   0.18). When comparing genetic distance within and between mitochondrial clades, values within

368   clades overlapped greatly with values observed among all samples between clades (Fig 7).

369   Fig 7. Plots of pairwise genetic distance (1 - proportion of shared alleles) against Euclidean

370   distance (km) showing positive isolation by distance. Solid lines show the predicted pattern of

371   isolation by distance from a linear mixed-effects model with maximum-likelihood population

372   effects (MLPEs; [52]). (A) Pairwise distances within Atlantic clade samples; (B) pairwise

373   distances within Atlantic clade samples (gray circles) and among samples from both Atlantic and

374   Gulf clades (white circles); (C) pairwise distances within Gulf clade samples; (D) pairwise

375   distances within Gulf clade samples (gray circles) and among samples from both Atlantic and

376   Gulf clades (white circles).

377

Morphological analysis

379   We observed four categories of temporal scales from both Atlantic and Gulf lineages

380   (Table 1). In 22% of specimens, temporals conformed to the 2+2 formula that Krysko et al. [16]

381   described as being invariant (Fig 1A). We found that 38% of specimens exhibited an extra

382   ventral temporal (Fig 1B), 26% of specimens had extra dorsal and ventral temporals (Fig 1C),

383   and 14% of specimens exhibited an extra dorsal temporal (Fig 1D). The frequency with which

384   these four categories occurred differed between Atlantic and Gulf lineage specimens (Table 2; df

385   = 3; Fisher's Exact P = 0.006), with Atlantic lineage snakes tending to have two dorsal temporals

386   and Gulf lineage snakes tending to have three dorsal temporals. Head shape, based on ANCOVA

387   of head width on head length, did not differ between Atlantic and Gulf lineages in either slope

388   (Fig 8; F = 0.07; df = 1; P = 0.79) or intercept (F = 0.48; df = 1; P = 0.49). Length of the dorsal

389   posterior temporal, expressed as a proportion of head length, differed significantly among

390   temporal categories (F = 18.34; df = 3; P < .0001), with the dorsal posterior temporal being

391   proportionately shorter when three dorsal temporal scales are present relative to when two dorsal

392   temporal scales are present (Fig 9).

393   Table 1. Frequency of occurrence of two and three dorsal temporal scales (DTs) between the

394   Atlantic and Gulf lineages of Eastern Indigo Snakes.

Mitochondrial Clade	Scale Condition			
	2+2	3_v+2	3_d+2	4+2
Atlantic	14	30	11	13
Gulf	5	1	2	9
Atlantic x Gulf	2	5	1	4
Atlantic x Unknown	2	6	1	4
Unknown	1	2	1	0

395

396   Fig 8. Bivariate plot of head height on head length. Values from Atlantic lineage indicated by

397   solid circles and solid line; values from Gulf lineage indicated by open circles and dashed line.

398   Fig 9. Box and whiskers plot of distribution of ratio of dorsal posterior temporal scale length to

399   head length in four categories of temporal scales (see Fig 1). Vertical lines indicate range; box

400   indicates interquartile, horizontal line indicates median; open diamond indicates mean.

401   When the length and width of the 6th and 7th infralabial scales were converted to a length-

402   to-width ratio, the distribution of our sample of scales from Atlantic lineage snakes encompassed

403   values for both type specimens for each scale (Fig 10). Length-to-width ratios differed between

404   6th and 7th infralabials (t = 8.07; df = 12, P < .0001), with 7th infralabials being more elongate

405   than 6th infralabials.

406   Fig 10. Distribution of length-to-width ratio of 6th and 7th infralabials in 11 Atlantic lineage

407   specimens of *Drymarchon couperi* (dark spots). Open triangles indicate ratios from type

408   specimen of *D. couperi* (Atlantic lineage); open diamonds indicate ratios from type specimen of

409   *D. kolpobasileus* (Gulf lineage).

410

Discussion

Gene sequence analysis

413   We found no variation of the nuclear NT3 locus in sequences generated by Krysko et al.

414   [15]. These authors included this locus because it "potentially represents an informative, single-

415   copy, unlinked locus that is likely evolving at a different rate than mtDNA (mitochondrial)

416   genes" (p. 114). From this we infer that the authors expected this nuclear gene to corroborate

417   patterns generated from mtDNA, solidifying their conclusion that two species are present.

418   However, if the locus was expected to be informative (i.e., *sensu* [57]), then the fact that it is

419   invariant indicates that it (1) does not support population structure consistent with two genetic

420   populations (i.e., two species), (2) was not necessary to include it in the study, and (3) only

421   served to restrict phylogenetic inference to the mitochondrial genome and its inherent problems.

422   From a statistical perspective, the only effect of including NT3 in their analysis was a slight

423   reduction of branch lengths due to the sequence's invariance. For these reasons, we suggest that

424   the gene sequence data do not provide compelling evidence in support of two distinct species of

425   *D. couperi* (*sensu lato*).

426

Microsatellite analysis

428   Our population genetic analyses found evidence of population structure within Eastern

429   Indigo Snakes, particularly along a north-south gradient and between southern Georgia and

430   peninsular Florida. However, patterns of microsatellite genetic structure reveal substantial

431   differences from the phylogenetic structure inferred from Krysko et al. [15]. First, the geographic

432   pattern of our population clusters suggests a north-south orientation rather than the Gulf-Atlantic

433   orientation suggested by mtDNA. Second, and most importantly, our Structure and sPCA

434   analyses document widespread contemporary admixture of alleles from the northern and

435   southern populations. Admixture occurs across the entire range of *D. couperi* (*sensu lato*) and

436   cannot be characterized as a narrow hybrid zone. While our MLPE analyses found support for

437   isolation by distance both across all samples and within each mtDNA clade, those analyses also

438   found high degree of overlap in genetic distance within and between clades, an observation that

439   we view as inconsistent with a two-species hypothesis. Further, our sPCA results, which

440   accounted for spatial autocorrelation among samples, are consistent with isolation by distance

441   along a north-south gradient and are not consistent with the statement by Krysko et al. [15] that

442   the two lineages of have "near discrete geographic distributions" (p. 118) and that "dispersal

443   between lineages is too low to influence demographic processes" (p. 119). Rather, our results

444   indicate high levels of contemporary gene flow, and we instead suggest that genetic structure

445   among populations is best described as continuous isolation by distance rather than discrete

446   evolutionary lineages. While isolation by distance is possible across two discrete clusters [58],

447   multiple analyses of genetic variation within our microsatellite data, including our sPCA which

448   accounts for spatial autocorrelation among samples, nevertheless did not correspond to the

449   genetic lineages recognized by Krysko et al. [15,16]. Together, we interpret the microsatellite

450   results as failure to support the presence of two discrete evolutionary metapopulations with little

451   or no admixture, the necessary criterion for the existence of two species [20]. Thus, we view

452   these data as sufficient to reject the two-species hypothesis, and we suggest the available data are

453   most consistent with the existence of a single evolutionary species, *D. couperi*.

454   The North American Coastal Plain is a global biodiversity hotspot [59]. In particular,

455   peninsular Florida has a high proportion of endemic species (e.g., snakes: [60–62]), which are

456   likely a product of refugial isolation on islands during periods of elevated sea level in recent

457   epochs and contemporary drainage-driven endemism [63,64]. However, our case study with *D.*

458   *couperi* adds to a growing number of examples of Florida organisms that appear, based on

459   modeling of one or few genetic loci, to represent species that are distinct from other mainland

460   counterparts, but for which microsatellite or similar data demonstrate substantial gene flow.

461   Burbrink and Guiher [60] estimated that there was such low gene flow between cottonmouths

462   (*Agkistrodon piscivorus*) in Florida and the mainland that speciation must have occurred between

463   those two regions, a hypothesis immediately contested by data from Strickland et al. [65] who

464   detected a broad geographic range of admixture using AFLP markers. Similarly, Thomas et al.

465   [66] described alligator snapping turtles (*Macrochelys temminckii*) from the Apalachicola River

466   and adjacent rivers to be a distinct species, despite microsatellite data from Echelle et al. [67]

467   that are inconsistent with this conclusion [68]. In a similar example, Gordon et al. [69] used

468   mtDNA to describe a population of *Anaxyrus boreas* in the western United States as a distinct

469   species, but this conclusion was contested by microsatellite analysis of gene flow [70].

470   We believe this emerging pattern along with a concept of species as evolutionary

471   metapopulation lineages [20] can guide us toward a more rigorous framework for species

472   delimitation. While analyses of one or few genetic loci can be informative by revealing apparent

473   monophyly, they should not be viewed as sufficient to diagnose and delimit species, because of

474   high error rate associated with the gene tree/species tree problem [24,25]. This issue is

475   particularly acute when relying on mtDNA [4,26]. We suggest that authors be required to meet

476   the necessary criterion of the unified species concept – that species are independently evolving

477   metapopulations lineages – by demonstration of little or no contemporary gene flow across

478   lineage populations using a multi-locus or genomic dataset. We note that Krysko et al. [16] also

479   used the unified species concept [20] as a framework to define species; while details about how

480   they operationally diagnosed species were not clear, it appears that they relied on evaluation of

481   reciprocal monophyly of mtDNA. While monophyly can be used as secondary criterion under

482   the metapopulation lineage concept [20], for the reasons outlined above we suggest authors

483   should evaluate monophyly in conjunction with analyses of contemporary gene flow and

484   population admixture, particularly when monophyly is evaluated with small numbers of loci.

485

486   Morphological analysis

487   Contrary to the results presented in Krysko et al. [16], we rejected the hypothesis that the

488   Atlantic and Gulf lineages are identifiable entities revealed by morphology. We reached this

489   conclusion after re-examining the variables used by Krysko et al. [16] to diagnose each lineage.

490   Of the disparities that emerge between our analyses and theirs, the conformation of the

491   infralabials is the most problematic. The figures presented by Krysko et al. [16] for the 6th

492   infralabial show great promise for diagnosing lineages, and separation of the lineages along PC1

493   of their analysis seems to provide statistical support for this character. However, we were struck

494   by how dissimilar Atlantic specimens appeared to be from the scale shape ascribed to them by

495   Krysko et al. [16]. Our analyses demonstrate that the 6th and 7th infralabials differ in shape, that

496   the shape of the 7th infralabial conforms to the shape ascribed to the Atlantic lineage, and that the

497   shape of the 6th infralabial conforms to that ascribed to the Gulf lineage. It is unclear which of

498   these scales was measured by Krysko et al. [16] and we found that the range of variation of each

499   scale within a sample of Atlantic lineage snakes encompasses both type specimens. One potential

500   explanation for this discrepancy is that Krysko et al. [16] intended to measure the 7th infralabial

501   but inadvertently measured the 6th for Gulf lineage specimens and the 7th for Atlantic lineage

502   specimens, perhaps because the mental scale sometimes was included in counts and other times

503   was not. Otherwise, we are left with a PCA that separates lineages based on one of the two

504   possible scales, but a univariate analysis that fails to confirm these lineages.

505   Our results for the temporal scale reveal great variation in the number of these scales

506   present in Eastern Indigo Snakes. The four categories that characterize this variation are found in

507   both Atlantic and Gulf lineage snakes, indicating that this feature is not diagnostic. Nevertheless,

508   Atlantic lineage snakes tend to have two dorsal temporals, while Gulf lineage snakes tend to

509   have three. We assume that Krysko et al. [16] intended to measure the dorsal posterior temporal

510   and, therefore, we focused our attention on this scale. Our data indicate that the length of the

511   dorsal posterior temporal, relative to head length, becomes shortened if three dorsal temporals

512   are present and becomes elongate if two dorsal temporals are present. This finding indicates that

513   the scale shapes revealed by PC2 of Krysko et al. [16] represent distinguishable groups, but these

514   represent two phenotypes and not two species. We infer that the different morphologies of the

515   dorsal posterior temporal result because, during embryonic development of some individuals, the

516   dorsal anterior temporal divides, limiting space for development of the dorsal posterior temporal.

517   Krysko et al. [16] also used head shape to diagnose the two lineages. Relative head

518   length and head height did load heavily on their PC1 axis and they used this to diagnose the

519   Atlantic lineage as having an elongate wide head and to diagnose the Gulf lineage as having a

520   short narrow head. Our bivariate examination of head length and height revealed no difference in

521   head shape between the two lineages. We are unsure why our results differed from Krysko et al.

522   [16], although we note that snake morphology can be difficult to measure consistently [71].

523   Specimens preserved with mouths open are likely to have larger values for head height than

524   those with mouths closed. If the relative frequency of open-mouthed versus closed-mouthed

525   specimens differed between lineages, this might yield a spurious association of head shape with

526   lineage. Our measurements were made from live specimens with closed mouths, which we

527   assume reduces measurement error. If the lineages truly differ in head shape, our ANCOVA

528   should have revealed this difference.

529

530   Natural history considerations

531   Our examination of genetic and morphological variation in *D. couperi* (*sensu lato*)

532   demonstrates that the two-species hypothesis proposed by Krysko et al. [16] is not supported by

533   available data. We offer several explanations for why true patterns of gene flow might result in a

534   lack of genetic and morphological differentiation. First, *D. couperi* movements can be extensive,

535 especially for males. Male annual home range size is as large as ca. 1500 ha [72] and average ca.

536 2.5–6.6 times larger than for females [72,73]. In fact, the disparity between male and female

537 home range sizes becomes exacerbated in large snakes, a feature dominated by data from *D.*

538 *couperi* (Fig 11; S3 Table). Within peninsular Florida, male *D. couperi* can move up to ca. 2 km

539 in a single day and average movement distance in males is approximately twice that of females

540 [38, D. Breininger, unpublished data]. Furthermore, males within peninsular Florida increase

541 their movement frequency, distance, and home range size during the breeding season [38,74].

542 Dispersal distance of males may be 10 times that of females [75], with a small adult male in

543 southern Georgia dispersing at least 22.2 km (straight line) over approximately two years [50].

544 Fig 11. Bivariate plot of female home range area (ha) on male home range area for 25 studies of

545 21 species of snakes (S3 Table). Open circles are data from *Drymarchon couperi*; closed circles

546 are data from all other species of snakes. Dashed line is null expectation if male and female

547 home range sizes are equal in size.

548 A second feature of *D. couperi* life history that reduces opportunity for speciation is the

549 variety of habitats used by these animals throughout a year (e.g., [72]), particularly in peninsular

550 Florida, where individuals will utilize habitats with varying degrees of anthropogenic disturbance

551 [76]. This broad habitat use reduces the opportunity for ecological barriers to gene flow and is

552 consistent with the high rates of gene flow indicated by our microsatellite analysis. Additional

553 life history observations show that *D. couperi* (*sensu lato*) can cross fresh and saltwater features

554 6–264 m wide [77; D. Stevenson, personal observation; D. Breininger, unpublished data], and we

555 note that traditional river barriers [63] do not appear to limit gene flow in these large snakes.

556 While we do not doubt that a historical climatic event may have separated *D. couperi* into two

557 populations [15], the observed levels of contemporary gene flow indicate that genetic

558   populations of *D. couperi* are in the process of merging back into a single evolutionary

559   population (i.e., species), as has been observed for diverse taxa following climatic cycles [78].

560   Together, our knowledge of *D. couperi* movement patterns and life history allows us to

561   hypothesize that limited female movement drives structure of the maternally-inherited mtDNA

562   data presented by Krysko et al. [15], even though high levels of male movement drive extensive

563   gene flow of the nuclear genome [79]. This life history-based model of intersexual variance in *D.*

564   *couperi* gene flow is consistent with two other reptile systems for which life-history strategies

565   generate contrasting patterns of gene flow among populations. First, female philopatry of

566   Loggerhead Sea Turtles (*Caretta caretta*) causes structuring of mtDNA in the Atlantic Ocean,

567   but high male dispersal drives significant nuclear gene flow among populations [80]. Second, a

568   recent study of Neotropical snakes found active-foraging species to have greater rates of nuclear

569   gene flow than ambush-predator species with more limited dispersal [81]. Last, and most

570   relevant, many squamates are polygynous and are characterized by greater male dispersal

571   relative to females [Fig 11; 82–85]; therefore, squamates typically have greater nuclear gene

572   flow than that of the mitochondrion [79]. This pattern is also seen in waterfowl [28,30]. Thus, we

573   suggest that *D. couperi* is similar to other reptile species in that life history provides explanations

574   for why phylogeographic patterns from mtDNA are inconsistent with historical and/or

575   contemporary patterns of nuclear DNA. Because of this incongruence and the high levels of

576   recent gene flow among populations observed by our microsatellite analysis, we place

577   *Drymarchon kolpobasileus* into synonomy with *D. couperi*. For now, we retain all Eastern

578   Indigo Snakes within *D. couperi*, but note that the analysis of Krysko et al. [16] reveals that this

579   species may not be diagnosable from *D. melanurus erebennus*.

580

Conservation implications

581

582   Eastern Indigo Snakes are now being released into the Conecuh National Forest of south-

583   central Alabama and to the Apalachicola Bluffs and Ravines Reserve in the panhandle of

584   Florida. Efforts to repatriate the species are the result of extensive collaborations and

585   partnerships between state and federal agencies, as well as non-governmental conservation

586   organizations. Initially, snakes used for release were to come from wild-caught gravid females

587   taken from sites in southeastern Georgia, retained in captivity until they laid eggs, and then

588   released at the point of capture. Eggs from these initial females then were to be hatched in

589   captivity and raised for release at one of the two repatriation sites or donated to the Orianne

590   Center for Indigo Conservation, where captive breeding from these and other donated animals

591   was to occur, eventually replacing use of wild-captured females.

592   Those charged with implementing conservation planning for *D. couperi* welcome the

593   opportunity to base those plans on the best published science. Many meetings were held among

594   stakeholders and scientists, and, as Krysko et al. [15] noted, their preliminary molecular results

595   [86] were presented at one of these early meetings as a challenge to the release of Atlantic

596   lineage snakes at the two release sites. Here we describe why data from that preliminary report

597   did not compel changes in the repatriation plan, and why continuation of that plan is unlikely to

598   encounter the problems predicted by Krysko et al. [15].

599   A lack of apparently viable populations of Eastern Indigo Snakes in the Florida

600   panhandle and southern Alabama and Mississippi was recognized as a significant gap in efforts

601   to retain Eastern Indigo Snakes across its historic geographic range. This gap guided the choice

602   of the two sites where repatriation was deemed reasonable [13]. Data from Krysko et al. [86]

603   were used to propose that only snakes of the Gulf lineage be used for repatriation because

604   genetic information from two isolated samples from the panhandle of Florida were of this

605   lineage. However, because Eastern Indigo Snakes were extirpated at both sites, discussions

606   centered on how to deal with the uncertainty of which lineage historically occurred at each site

607   and, therefore, which lineage(s) were appropriate to release at each site. This uncertainty

608   recognized the fact that, for both sites, the 3rd and 4th closest localities currently occupied by

609   Eastern Indigo Snakes have the Atlantic lineage. Additionally, the Atlantic lineage occurs in the

610   Suwannee drainage, which empties into the Gulf of Mexico, further illustrating the uncertainty in

611   identifying the location of the Gulf-Atlantic split and that both lineages are found in north

612   Florida where they might serve as colonists for the release sites. Participants in these discussions

613   were made aware of then-unpublished microsatellite data demonstrating widespread gene flow

614   between lineages and observations from the zoo and herpetocultural communities documenting

615   interbreeding of the two lineages with no apparent effects of outbreeding depression. While there

616   is currently no evidence of ecological differences between Atlantic and Gulf lineage snakes,

617   snakes from northern populations are known to differ markedly from southern populations in

618   seasonal activity. Specifically, *D. couperi* in the latter region do not appear dependent upon

619   Gopher Tortoise (*Gopherus polyphemus* Daudin 1802) burrows for winter refugia, likely because

620   of milder winter temperatures [38,72,73,87,88]. As a result, use of snakes from similar latitudes

621   appeared to be more important in ensuring successful repatriation than use of snakes from the

622   same mitochondrial lineage. The majority of meeting participants therefore concluded that the

623   wide movements of male *D. couperi* and broad habitat use of the species made matching of

624   mitochondrial DNA lineages less important in guiding repatriation than matching spatial

625   variation in ecology. Preservation of mitochondrial lineages was already achieved through

626 apparently viable populations at the northern and southern geographic extremes of *D. couperi*

627 and release of snakes of one mitochondrial lineage into an area historically occupied by the other

628 lineage was viewed simply as replicating patterns of gene flow observed throughout much of

629 north and central Florida.

630 Because inferences from phylogenetic analyses of few loci can conflict with

631 microsatellite-based analyses of contemporary admixture, we suggest that, at a minimum, both

632 tools should be considered to adequately reveal recently diverged taxa. Additionally, we argue

633 that a more-consistent voice from the community of systematists will emerge when life-history

634 data are allowed to inform speciation models. For example, the potential for large snakes to

635 move long distances as well as a difference in movement behavior between the sexes limit the

636 utility of mitochondrial data alone to reveal novel species. We suspect this disparity in

637 movement is rampant within squamates (e.g., [89]). Finally, species descriptions for which

638 diagnoses merely report measures of variables that overlap widely among taxa (e.g., [16,66]) or

639 that are not derived from results presented in the paper (e.g., [60]) should be rejected because

640 they fail to provide further evidence that proposed taxa are identifiable as individuals. We point

641 to papers that carefully meld phylogenetic and population genetic analyses [70,90,91] as

642 improved examples of the process by which researchers might determine whether several

643 populations have diverged sufficiently to be recognized as distinct species. Last, we suggest

644 authors and reviewers be particularly critical of species descriptions without careful analysis of

645 admixture or contemporary gene flow, because these papers may incorrectly delimit species,

646 contribute to erroneous hyperdiversity, and confuse efforts to understand and conserve imperiled

647 biodiversity.

648

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660

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662 **Conceived and designed the experiments:** BF JB SS CG.

663 **Project coordination:** BF.

664 **Contributed reagents/materials/analysis tools:** JB SS CJ CG.

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667 **Wrote the paper:** BF JB SS DS JRO DAS CG.

668

Supporting Information

670 S1 Fig. Results of spatial principle components analysis using a Delaunay triangulation
671 connection network. The eigenvalues (left) for each axis where positive values indicate global
672 structure and negative values indicate local structure, and the Moran's *I* (right) plotted against
673 the variance for each axis.

674 S2 Fig. Additional results of spatial principle components analysis using a Delaunay
675 triangulation connection network demonstrating the spatially lagged scores from the first (A) and
676 second (B) axes. Atlantic clade samples are displayed using circles while Gulf clade samples are
677 displayed using squares. Samples with more extreme values/colors are more genetically
678 differentiated.

679 S3 Fig. Results of spatial principle components analysis using a distance-based connection
680 network where samples are considered connected if they are within 22.2 km which is the
681 maximum recorded dispersal distance for Eastern Indigo Snakes. The left figure shows the
682 eigenvalues for each axis where positive values indicate global structure and negative values
683 indicate local structure and the right figure shows the Moran's *I* plotted against the variance for
684 each axis.

685 S1 Table. GenBank accession numbers for sequence data from the nuclear gene *neurotrophin*-3
686 (NT3) from Krysko et al. [15] that were analyzed to generate Fig 2.

687 S2 Table. Multiplex PCR panels for *Drymarchon couperi* microsatellite loci. The names of loci
688 are as in [39].

689 S3 Table. Citations for snake studies (N = 28) describing intersexual variance in home range size
690 (hectares) for 22 species of snakes used to generate Fig 9. See [92] for an earlier review of the
691 topic.

692

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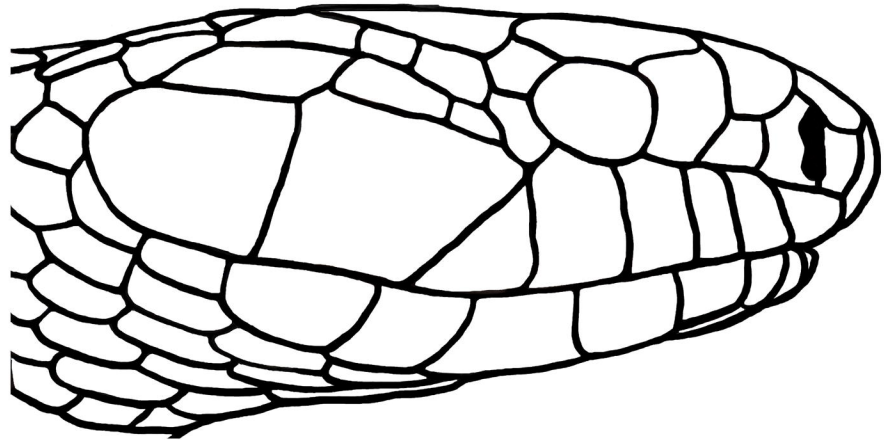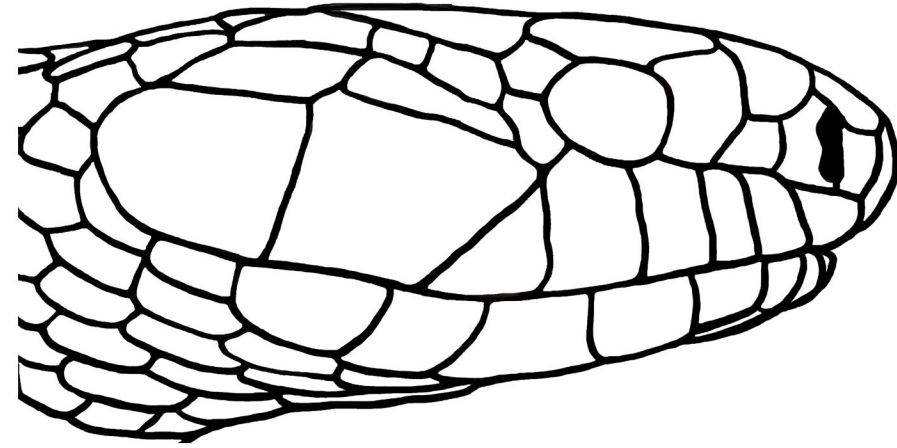
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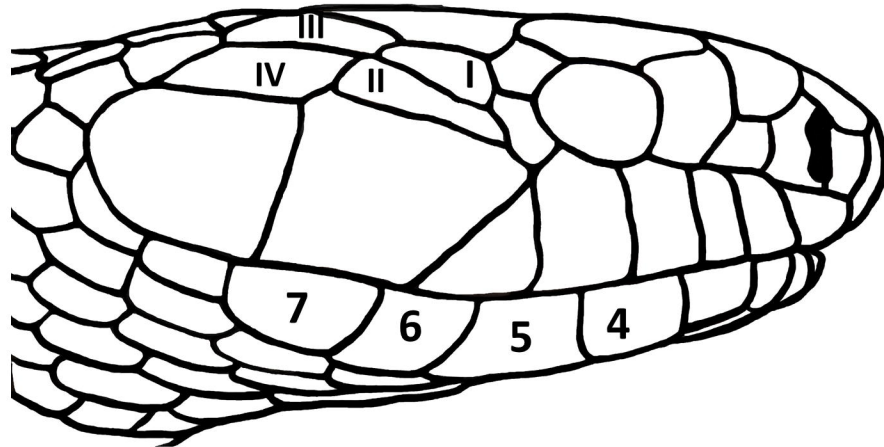
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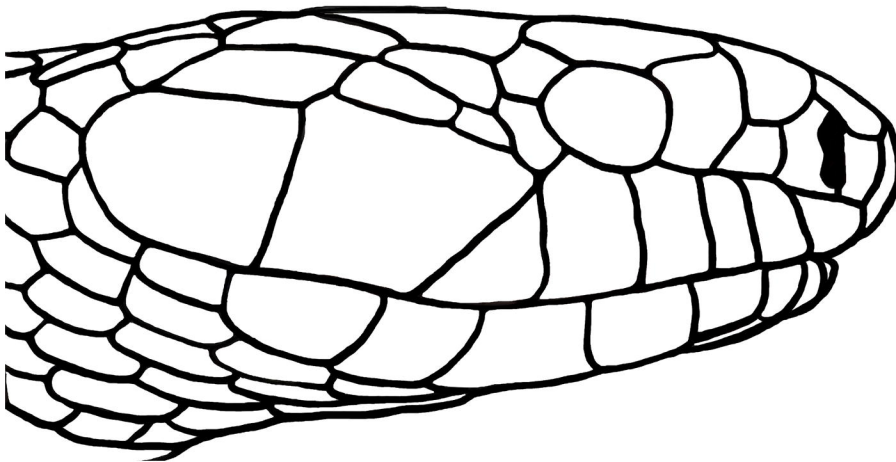
C

D

Masticophis flagellum
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First Axis
- ○ -1.00 – -0.50
- ◐ -0.50 – -0.20
- ◑ -0.20 – 0.20
- ◕ 0.20 – 0.60
- ● 0.60 – 1.00

Second Axis
- ○ -0.45 – -0.20
- ◐ -0.20 – 0.00
- ◑ 0.00 – 0.20
- ◕ 0.20 – 1.00
- ● 1.00 – 1.20