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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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30 **Abstract**

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

46

47 **Introduction**

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

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

115 **Materials and Methods**

116 **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

159 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 μ M multiplexed
175 primer mix (each primer at equal concentrations), and 1 μ l of DNA extract in a total volume of 7
176 μ l. The PCR protocol was modified from Shamblin *et al.* [39] for multiplex PCR and consisted
177 of an initial denaturation of 95°C 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 $1 - \text{proportion of shared alleles}$ [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

233 Morphological analysis

234 Krysco 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 Krysco 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 Krysco
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 Orianna 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

302 **Results**

303 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

317 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

378 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.

<u>Mitochondrial Clade</u>	<u>Scale Condition</u>			
	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

411 **Discussion**

412 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

427 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. Breining, 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. Breining, 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 synonymy 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

581 Conservation implications

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

661 **Author Contributions**

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

663 **Project coordination:** BF.

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

665 **Collected the data:** BF JB SP MH CG.

666 **Analyzed the data:** BF JB SS CG.

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

668

669 **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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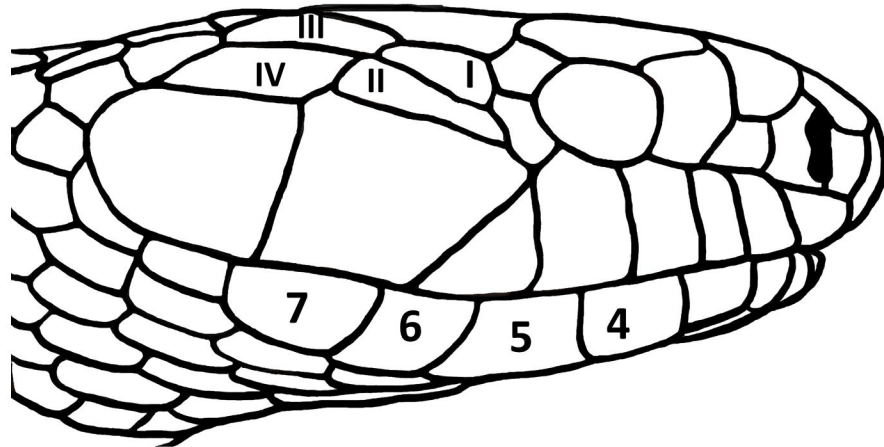
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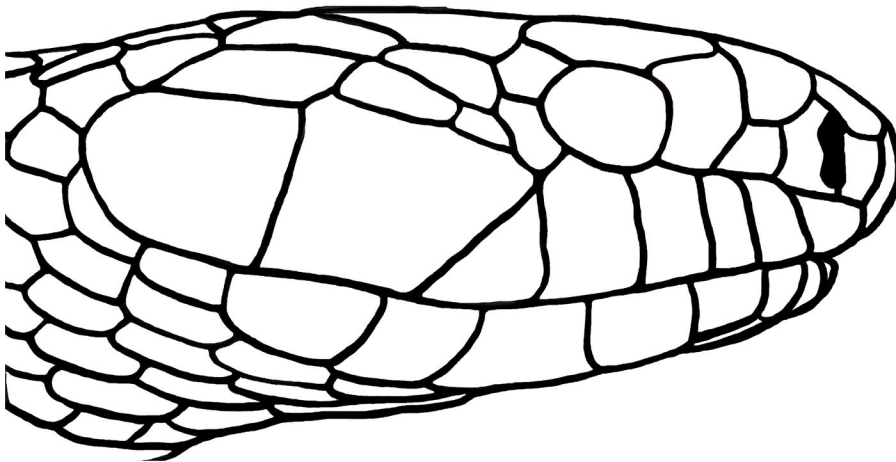
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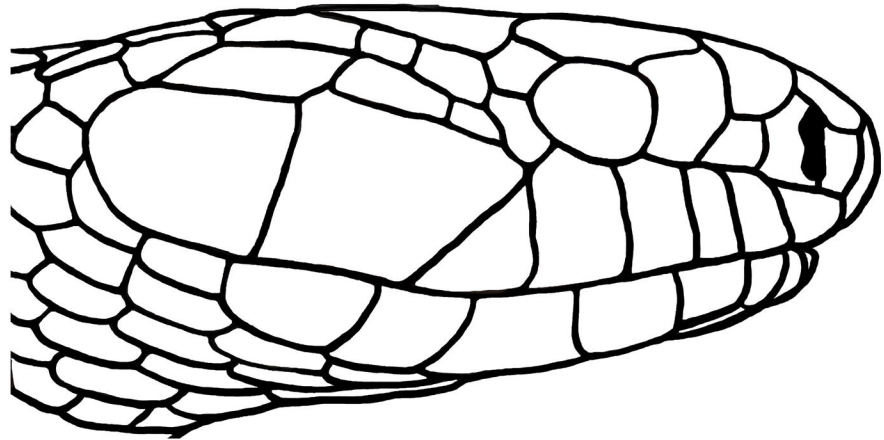
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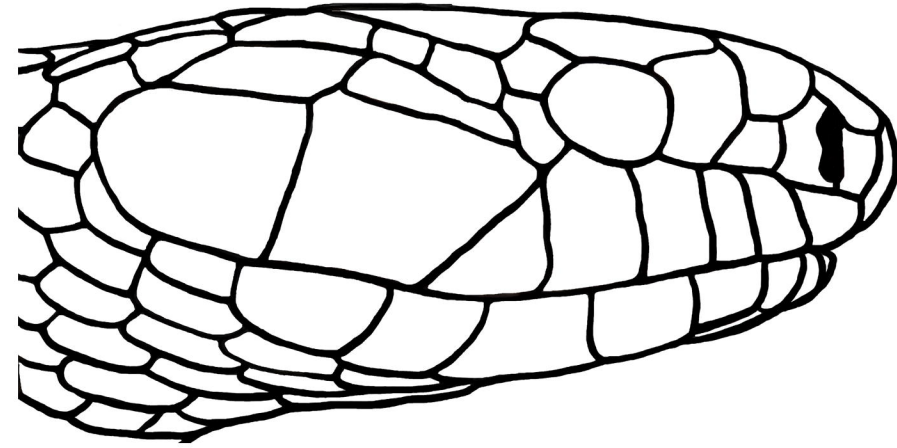
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Masticophis flagellum

Coluber constrictor

Drymarchon melanurus

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