Phylogenetic, population genetic, and morphological analyses reveal evidence for one species of Eastern Indigo Snake (*Drymarchon couperi*)

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Running title: Systematics of *Drymarchon couperi*

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 Krysko et al. (2016a) was based entirely on variance of mitochondrial DNA sequence data, (2) microsatellite data demonstrate significant nuclear gene flow between mitochondrial lineages and a clear isolation-

by-distance pattern across the species' entire range, and (3) morphological analyses recover a single diagnosable species. Our results reject recent conclusions of Krysko et al. (2016a,b) regarding species delimitation and taxonomy of *D. couperi*, and we 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.

ADDITIONAL KEYWORDS: Coastal Plain – Conservation – *Drymarchon couperi* – *Drymarchon kolpobasileus* –Gene Flow – Population Genetics – Repatriation – Taxonomy.

INTRODUCTION

Accurate species delimitation and description are critical not only for understanding global patterns of biodiversity, but also to guide effective conservation strategies (Avise, 1989; Agapow *et al.*, 2004; Mace, 2004; Frankham *et al.*, 2012). For example, species are often delimited into multiple species on the basis of systematic studies utilizing molecular genetic data, thereby requiring adjustment of existing conservation management plans (e.g., Daugherty *et al.*, 1990). When species delimitation methods fail to correctly diagnose individuals (*sensu* Ghiselin, 1997), such errors can have significant consequences for conservation and management of imperiled species by reducing or diverting finite conservation resources (Agapow *et al.*, 2004). Therefore, taxonomic division into multiple species should be performed carefully and only when robust evidence supports a decision to revise. Indeed, authors have cautioned that studies

of species delimitation should be conservative, because "it is better to fail to delimit species than it is to falsely delimit entities that do not represent actual evolutionary lineages" (Carstens *et al.,* 2013).

The Eastern Indigo Snake (*Drymarchon couperi* Holbrook, 1842) is a large colubrid of the Coastal Plain of the southeastern United States. However, *D. couperi* populations have declined precipitously over the last century, largely due to habitat loss, habitat fragmentation, and historical over-collecting for the pet trade (U.S. Fish and Wildlife Service, 1982, 2008). As a result of these declines, *D. couperi* is listed as Threatened under the U.S. Endangered Species Act (U.S. Fish and Wildlife Service, 1978, 2008). Potentially viable populations of *D. couperi* remain in large contiguous habitats in southeastern Georgia (Stevenson *et al.,* 2009; Hyslop *et al.,* 2011; Enge *et al.,* 2013), and throughout peninsular Florida (Breininger, Legare & Smith, 2004; Enge *et al.,* 2013), but the species was likely extirpated from Mississippi, Alabama, and the Florida panhandle (Enge *et al.*, 2013).

Current conservation management plans for *D. couperi* were developed under the hypothesis that *D. couperi* represents a single species. However, this hypothesis was recently challenged by Krysko *et al.* (2016a), who used DNA sequence analyses to describe two genetic lineages of *D. couperi* – an Atlantic lineage, including populations in southeastern Georgia and eastern peninsular Florida, and a Gulf lineage of populations in western and southern peninsular Florida and the Florida panhandle. This phylogeographic study was followed by a second paper (Krysko *et al.,* 2016b) that analyzed morphological variation between the Atlantic and Gulf lineages and provided an official description of the Gulf lineage as a purported novel species, the Gulf Coast Indigo Snake (*Drymarchon kolpobasileus* Krysko, Granatosky, Nuñez, Smith 2016).

Given the conservation status of *D. couperi* (*sensu lato*), these results have potentially important consequences for conservation of Eastern Indigo Snakes. First, division of *D. couperi* (*sensu lato*) into two smaller-ranged species results in two species with substantially smaller population sizes that are, therefore, at greater risk of extinction (*sensu* Agapow *et al.,* 2004; e.g., Pauly, Piskurek & Shaffer, 2007). Second, conservation and recovery of two rare species requires more time and funds than one; both resources are in short supply. Finally, as noted by Krysko *et al.* (2016a), active conservation management plans for *D. couperi* (*sensu lato*) include population repatriation projects in Alabama and the Florida panhandle, where populations attributed to the Gulf lineage presumably were extirpated. Repatriation projects should be informed by phylogeographic and genetic developments (Soltis and Gitzendammer, 1999). The description of *D. kolpobasileus*, therefore, causes increased logistical complexity for Eastern Indigo Snake captive breeding and repatriation projects.

We represent additional experts on Eastern Indigo Snake taxonomy, ecology and conservation, many of whom participated in an inter-agency workshop on Eastern Indigo Snake taxonomy referenced in Krysko *et al.* (2016a,b). During that event, consequences of the discovery that Eastern Indigo Snakes comprise two genetic lineages were debated, and this debate was used to inform conservation plans for the species. Skepticism was voiced that the two lineages represent diagnosable species based largely on description of microsatellite data documenting widespread admixture of the lineages. Here, we repeat that debate by using multiple approaches to evaluate the hypothesis that *D. couperi* comprises two distinct species (Krysko *et al.,* 2016b). First, we review mitochondrial and nuclear DNA sequence analyses presented by Krysko *et al.* (2016a) as well as morphological data presented by Krysko *et al.* (2016b) and describe limitations of those data and analyses. Second, we analyze a novel

93 microsatellite DNA dataset and test for evidence of contemporary gene flow between the two
94 genetic lineages identified by Krysko *et al.* (2016a). Third, we provide new analyses of
95 morphological data collected from 125 Eastern Indigo Snakes, including individuals from both
96 genetic lineages of Krysko *et al.* (2016a). Specifically, we evaluate the diagnostic features of
97 head and scale shape presented by Krysko *et al.* (2016b). Last, we review features of the life
98 history of *D. couperi* and suggest how they inform interpretations of genetic data.
99

100 # REVIEW OF PUBLISHED DATA

101 ## GENE SEQUENCE ANALYSIS

102 To infer evolutionary history among populations of *D. couperi* (*sensu lato*), Krysko *et al.*
103 (2016a) analyzed sequence data obtained from three genetic markers: the mitochondrial
104 (mtDNA) genes cytochrome *b* (CytB) and nicotinamide adenine dinucleotide dehydrogenase
105 subunit 4 (ND4) and the nuclear gene neurotrophin-3 (NT3). Because CytB and ND4 are linked
106 components within the non-recombinant and maternally-inherited mitochondrial genome, we
107 note that those two markers are linked on a single locus. The authors estimated phylogenetic
108 relationships among populations by analyzing a concatenated dataset including both
109 mitochondrial and nuclear loci. These data were evaluated with maximum likelihood (ML) and
110 Bayesian analyses; for the Bayesian analysis, the dataset was partitioned such that nucleotide
111 substitution was modeled separately for each locus. Because both analyses generated similar
112 phylogenetic hypotheses, the authors described results only from the Bayesian analysis.
113 Phylogenetic analyses of a single or few genetic loci frequently describe evolutionary
114 patterns that do not reflect the organism's true evolutionary history (i.e., the gene tree/species
115 tree problem; Avise *et al.,* 1983; Funk, 1999; Funk and Omland, 2003; Dupuis, Roe & Sperling,

2012). In particular, use of and reliance on mtDNA for phylogenetic and taxonomic analyses has been criticized because mtDNA has a vastly different natural history than the primary mode of genetic inheritance, nuclear DNA (nDNA). Mitochondrial DNA has a lower effective population size, higher mutation rate, and frequently defies critical assumptions of neutral evolution by being under selection (Ballard & Whitlock, 2004; Frankham *et al.*, 2012). More importantly, mtDNA is maternally inherited and, therefore, may not describe an organism's true patterns of inheritance expressed through the nuclear genome (Ballard & Whitlock, 2004). This is particularly problematic for species with relatively low dispersal rates that are more likely to show phylogeographic breaks that are not driven by decreased gene flow but by chance alone (Irwin, 2002), or for species with intersexual differences in movement, site fidelity, or breeding behavior (Scribner *et al.*, 2001; Thorpe, Surget-Groba & Johannson, 2008; Peters, Bolender & Pearce, 2012).

Given these and other limitations, a customary practice in phylogenetic studies is to use both mitochondrial and nuclear loci and to describe phylogenetic patterns inferred from these two components of the genome separately (e.g., Irwin, 2002; Funk & Omland, 2003; Gamble *et al.*, 2008; Grismer *et al.*, 2014). This practice can help identify situations for which phylogenetic hypotheses generated from mtDNA (1) are incongruent with hypotheses from the nuclear genome and that (2) might be erroneously assumed to accurately depict the species tree. However, Krysko *et al.* (2016a) combined the mitochondrial and nuclear markers and used that concatenated dataset to infer both ML and Bayesian phylogenies from the combined datasets. Because mutation rates of mtDNA are higher than those of nDNA, phylogenetic analysis of the concatenated data will be driven by the more variable markers from the mtDNA, thus leaving their results subject to all the criticisms associated with mtDNA data (Ballard & Whitlock, 2004;

Frankham *et al.*, 2012). We do note that the fundamental differences between mitochondrial and nuclear DNA can be leveraged to understand interesting evolutionary history of study organisms (e.g., introgressive hybridization; Rubinoff & Holland, 2005; Grismer *et al.*, 2014; Folt *et al.*, 2016). However, we also note that the analyses summarized above do not exploit these potential benefits (i.e., by comparing mitochondrial and nuclear gene trees), and we instead argue that both ML and Bayesian analyses of the Eastern Indigo Snake data are biased toward describing patterns from maternally inherited mtDNA.

Krysko *et al.* (2016a) used mixed-model Bayesian inference of the sequence data to infer divergence dates, population size, and migration rates over time. These authors hypothesized that the two lineages of Eastern Indigo snakes are derived from an ancestor that colonized the southeastern United States via eastward migration of *D. melanurus* along the Gulf Coast (Auffenberg, 1963). In an attempt to understand migration and gene flow between mitochondrial clades, Krysko *et al.* (2016a) described migration rates inferred from population genetic summaries of their mitochondrial analysis; the authors suggested those rates were too low to allow for homogenization of the two populations. However, estimates of migration rates between clades were derived from the same data used to define those clades. A better approach, analogous to model validation (McGarigal, Cushman & Stafford, 2000), would have been to estimate the clades and migration rates between them using two independent data sources. We suggest the authors' analysis of migration greatly limits their ability to delimit species and is problematic because coalescent theory would suggest the mitochondrial clades found by the authors would also be expected if all animals were within a single species. In a population of constant size, the two deepest branches of a gene tree are expected to comprise approximately half of the gene's history from present to the most recent common ancestor (Hudson, 1990;

Wakely, 2008). Furthermore, if an individual is more likely to share a more-recent common ancestor with geographically proximate individuals, these two clades, predicted by coalescent theory, will often display geographic separation (Irwin, 2002). Thus, the two mitochondrial clades that loosely correspond with a lengthy transect from Georgia to the southern tip of Florida provide only weak evidence of population structure, let alone evidence to suggest a species boundary.

Krysko *et al.* (2016b) found that individuals from the contact zone between the two lineages (e.g., Alachua, Clay, Indian River, and Volusia counties) are morphologically intermediate between what they considered core populations of the two mitochondrial lineages; the authors hypothesized that such intermediate populations might represent a "hybrid" zone between lineages, an implicit hypothesis of contemporary gene flow between the two mitochondrial lineage populations (Krysko *et al.,* 2016b). We also note that some counties possessing morphologically intermediate snakes were distant from the apparent contact zone between mitochondrial lineages (e.g., Hardee County), suggesting extensive gene flow. If contemporary nuclear gene flow is as extensive as this, then such evidence is a serious challenge to the conclusion that the two lineages represent separate species (Frankham *et al.*, 2012).

MORPHOLOGICAL ANALYSIS

Krysko *et al.* (2016b) conducted two sets of analyses designed to test whether morphological characteristics distinguish the two genetic lineages. First, they tested whether specimens from each lineage differed in seven morphological measurements traditionally used to summarize snake scalation (e.g., Savage, 2002): the mean/median number of ventral, subcaudal, supralabial, and infralabial scales; modal counts of temporal scales (traditional formula of dorsal

185 + ventral), ocular scales (pre- and postoculars); and dorsal scale row counts (one head length
186 posterior to head, midbody, and one head length anterior to cloaca). This set of analyses,
187 essentially, yielded no differences between lineages.
188 As a second set of analyses, Krysko *et al.* (2016b) performed principal components
189 analyses (PCA) on five linear measurements of head morphology: head length, head height,
190 length of a temporal scale, length of the 7th infralabial, and width of the 7th infralabial (but see
191 below). PCA was first run using specimens with known genetic lineage assignment and then
192 repeated including additional specimens for which genetic lineage assignment was not known.
193 We focus on the first analysis. Krysko *et al.* (2016b) standardized each variable by dividing each
194 measurement by the geometric mean of all measurements of that individual and log-transformed
195 each variable to normalize their distributions. Head length, head height, and 7th infralabial length
196 loaded positively and 7th infralabial width loaded negatively on the first principal component,
197 which explained 44.7% of variance. This axis separated specimens that were described to be
198 long- and wide-headed with long and narrow 7th infralabials (high PC1 scores; almost
199 exclusively Atlantic lineage snakes) from specimens that were described to be short and narrow-
200 headed with short and wide 7th infralabials (low PC1 scores; almost exclusively Gulf lineage
201 snakes). Temporal length loaded heavily and positively on the second principal component,
202 which explained 25.4% of variance and separated specimens with relatively elongate temporals
203 (high values on PC2; primarily Atlantic lineage) from specimens with relatively short temporals
204 (low values on PC2; primarily Gulf lineage). The authors then performed a discriminant function
205 analysis (DFA) to determine how well the morphological variables assigned specimens to their
206 correct genetic lineage. Because DFA assigned 96.3% of specimens to their correct genetic
207 lineage, Krysko *et al.* (2016b) used Wards hierarchical cluster analysis to infer lineages for

specimens that were not included in the genetic analysis; no details were provided indicating how such assignments were made. Finally, Krysko *et al.* (2016b) examined contact between the 5^{th} and 7^{th} supralabial scales, demonstrating that this condition characterized all members of the Atlantic and Gulf lineages of *D. couperi* (*sensu lato*), but that it also characterized 19% of Texas Indigo Snakes (*D. melanurus erebennus*).

We find serious inconsistencies in the scale characters measured as well as the methods used to evaluate those characters. Krysko *et al.* (2016b) did not clearly identify the specific temporal scale they measured. These authors described temporals to be 2+2 and invariant on each side, but noted that "many specimens had smaller divided scales" (Kryso *et al.*, 2016b, p. 555). The 2+2 formula specifies the presence of a pair of anterior (one dorsal and one ventral) and a pair of posterior (one dorsal and one ventral) temporals (Fig. 1A). This condition is illustrated in Krysko *et al.* (2016b) for *Drymarchon melanurus*, but occurs in none of their figures of *D. couperi* (*sensu lato*). Instead, figures for *D. couperi* (*sensu lato*) show an extra ventral anterior temporal (3_v+2 condition; Fig. 1B; likely the source of the phrase quoted above) or an extra dorsal and ventral anterior temporal (4+2 condition; Fig. 1C). When identifying the temporal scale they measured, Krysko *et al.* (2016b) highlight the central-most dorsal temporal of an individual expressing the 4+2 condition, but when summarizing results of their temporal scale analyses they highlight the dorsal posterior temporal of an Atlantic lineage individual expressing the 3_v+2 condition and the dorsal posterior temporal of a Gulf lineage individual expressing the 4+2 condition. Thus, it is unclear which temporal scale Krysko *et al.* (2016b) measured for specimens with only two dorsal temporals, and how measures of the central dorsal temporal allowed interpretation of unmeasured posterior temporals.

Examination of the infralabial character of Krysko *et al.* (2016b) reveals similar problems. They state that the 7th infralabial was used in their morphological analyses, but they highlight the 6th infralabial in the figures identifying the scale in question. This incongruity is evident because the 4th and 5th infralabials are noticeably enlarged in all Eastern Indigo snakes that we have examined (see results below), a feature consistent with all figures in Krysko *et al.* (2016b), and the highlighted scale is adjacent to the 5th infralabial. Thus, it is unclear which scale was measured for this important character and whether the same scale was measured among all individuals included in analysis. It is important to note that the mental scale is visible in all figures of Krysko *et al.* (2016b) and should not be included in identifying the position of individual infralabials.

Finally, we find problems with the DFA analysis used to determine rates of misclassification and the cluster analysis used to classify snakes for which no molecular data are available. Discriminant function analysis is a statistical technique used to generate linear functions of variables that separate previously-identified groups. Krysko *et al.* (2016b) did not publish these discriminant functions and instead used a clustering algorithm (Ward's hierarchical analysis) to classify snakes into one of the two lineages. These authors report that the discriminant model correctly identified 96.3% of specimens used to create the model; yet they apparently allowed data used to create the model to also be used to test it, a procedure that yields inflated estimates for fit of specimens to the model (SAS Institute, Inc., 2008). A better approach would have been a *k*-fold cross validation procedure in which the dataset is divided so that part of the data is used to generate the statistical model and the remainder is used for validation (McGarigal *et al.*, 2000). Nevertheless, DFA is an appropriate statistical tool for classifying specimens using morphological data when molecular data are not available. Unfortunately,

Krysko *et al.* (2016b) use Wards hierarchical cluster analysis instead. This agglomerative technique joins pairs of specimens that are most similar to each other based on measured variables, and then iteratively adds additional specimens that are progressively less similar to the initial clusters. However, Krysko *et al.* (2016b) did not report how they used the results of the clustering analysis to assign individuals to Atlantic and Gulf Lineages, although statistical approaches are available for testing for group membership and significance (McGarigal *et al.*, 2000). We suspect that a dendrogram was generated, the basal dichotomy was used to define two morphological groups, and these groups happened to conform to the Atlantic and Gulf lineages. If so, we find two serious problems with this method. First, cluster analyses are designed to estimate numbers of groups when group membership is unknown. Because the number of groups (two) was known, DFA was a more appropriate tool. Second, while a cluster analysis will always generate a dendrogram that includes all specimens and that dendrogram will have at least two groups defined by a basal dichotomy, the procedure also provides statistical tests of group membership from the terminal nodes to the basal node and tests whether two or more groups differ significantly. Thus, it is possible for some individuals appearing on a dendrogram to belong to no group (SAS Institute, Inc., 2008). We therefore suspect that some (perhaps many) lineage assignments made by Krysko *et al.* (2016b) using the clustering method lack statistical support for membership in their assigned lineages. We think this is especially likely for the Mississippi specimen, because it is a geographical outlier.

MATERIALS AND METHODS

GENE SEQUENCE ANALYSIS

To explore the extent to which nuclear sequence data support speciation within Eastern Indigo Snakes, we accessed the Krysko *et al.* (2016a) sequence data from GenBank (Appendix I) and used ML methods to infer a nuclear gene tree from the NT3 dataset, following the methods used by Krysko *et al.* (2016a). This dataset included 23 *D. couperi* (*sensu lato*) samples (N = 13 Atlantic clade, N = 10 Gulf clade) and four outgroup taxa (*Drymarchon melanurus erebennus*, *Drymarchon melanurus rubidus*, *Coluber flagellum*, *Coluber constrictor*). We fit different models of nucleotide substitution and ranked them using BIC; this procedure suggested that the Kimura (1980) model best fit the data. We then estimated a ML phylogeny for NT3 using the package 'phangorn' (Schliep, 2011) in the statistical Program R (R Core Team, 2016). This method allowed us to test whether a phylogenetic pattern inferred from nuclear DNA alone differed from a concatenated analysis of mitochondrial and nuclear DNA.

MICROSATELLITE ANALYSIS

We extracted and genotyped microsatellite DNA from 428 tissue samples (scale, shed skins, or muscle from road-killed individuals) throughout peninsular Florida and southern Georgia. Twenty-five samples were obtained from the collections of the Florida Museum of Natural History, including 20 samples used in Krysko *et al.* (2016a). The samples from Krysko *et al.* (2016a) included individuals from central Florida that represented both mitochondrial clades where they occur in close proximity. The remaining Florida samples (N = 170) were collected during field studies of *D. couperi* (Bauder & Barnhardt 2014, Bauder *et al.* 2016a) in and around Highlands County or opportunistically by authorized project partners. The samples from Georgia (N = 233) were collected by multiple project partners as part of a study of population fragmentation in the state (S. Spear *et al.*, unpublished data). Our samples include

similar representation of both mitochondrial lineages (55% Atlantic and 45% Gulf). We extracted DNA using the Qiagen DNeasy blood and tissue extraction kit (Qiagen, Inc., Valencia, CA). We ran 17 microsatellite loci (Shamblin *et al.*, 2011) within three multiplexed panels using the Qiagen Multiplex PCR kit (see Appendix II for details). Each reaction contained 1X Qiagen Multiplex PCR Master Mix, 0.2 µM multiplexed primer mix (each primer at equal concentrations), and 1 µl of DNA extract in a total volume of 7 µl. The PCR protocol was modified from Shamblin *et al.* (2011) for multiplex PCR and consisted of an initial denaturation of 95ºC for 15 min, 20 touchdown cycles of 94°C for 30 s, 60°C minus 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 s and 72°C for 1 min, and a final elongation step of 60°C for 30 min. Multiplexed PCR products were run on a 3130xl Applied Biosystems Genetic Analyzer at the University of Idaho's Laboratory for Ecological, Evolutionary, and Conservation Genetics. We scored fragment sizes using Genemapper 3.7 (Applied Biosystems).

We tested for the presence of null alleles that would lead to violations of Hardy-Weinberg equilibrium assumptions. We eliminated any loci that had an estimated null allele frequency > 0.10 using the software FreeNA (Chapuis & Estoup, 2007). We estimated population structure and number of genetic clusters using the Bayesian clustering algorithm Structure 2.3.4 (Pritchard, Stephens & Donnelly, 2000). We used the admixture model with 100,000 iterations following 10,000 burn-in repetitions. We evaluated K = 1–10 with five replicates for each value of K. We used Structure Harvester (Earl & vonHoldt 2012) to implement the Delta K method of Evanno *et al.* (2005) to estimate the number of clusters that best explain the microsatellite data. We used CLUMPP v.1.1.2. (Jakobsson & Rosenberg, 2007) to estimate the optimal cluster assignment in a single file based on the five replicates for the best

supported values of K. Because the Evanno *et al.* (2005) method analyzes changes in likelihood between values of K, it cannot estimate Delta K for K = 1; therefore, we assessed the probability of a K = 1 scenario with the raw likelihood values from Structure. Given biases of methods to estimate population structure from microsatellite data (Janes *et al.*, 2017), we sought to follow recommendations from Janes *et al.* (2017) by describing and comparing population structure predicted by both the Delta K and raw likelihood outputs from Structure, while also reporting bar plot outputs for different values of K supported by those methods. We used the program CLUMPAK (Kopelman *et al.*, 2015) to visualize bar plot outputs from Structure. We note that during these exploratory hierarchical analyses we observed additional fine scale genetic structure among populations; these details were outside the scope of the current analysis, but we intend to examine these data more fully in a future paper.

In addition to the clustering analyses, we used Mantel tests to test for isolation by distance both across and within the two mitochondrial clades. We had exact coordinates for all samples collected by co-authors. In the case of museum samples, points were only precise to approximately 10 kilometers. However, considering that our study extent ranged approximately 800 kilometers from north to south, we do not consider this lack of precision as a major source of error for our isolation-by-distance analysis. We estimated genetic distance at the individual level using proportion of shared alleles (Bowcock *et al.*, 1994). We estimated proportion of shared alleles using package ‘memgene’ (Peres-Neto & Galpern, 2014) and implemented Mantel tests using package ‘vegan’ (Oksanen *et al.*, 2016) in the statistical Program R (R Core Team, 2016).

MORPHOLOGICAL ANALYSIS

We examined 114 *D. couperi* (*sensu lato*) housed at the Orianne Center for Indigo Conservation (OCIC), a sample that included 68 and 17 specimens from the Atlantic and Gulf lineages of Krysko *et al.* (2016a), respectively, along with 4 specimens of unknown lineage assignment, 12 specimens derived from F1 hybrids of Atlantic and Gulf lineage snakes, and 13 hybrids of Atlantic lineage snakes crossed with adults of unknown origin. Lateral or dorsolateral photos of the head were taken of each specimen, including a millimeter ruler for scale. The photos were used to record the condition of the temporal scales for each specimen. Four categories were recognized based on the number and position of temporal scales (Fig. 1). We generated a contingency table providing counts of specimens in each of the four categories for each lineage, specimens of unknown lineage, and hybrids. Fisher's exact test was used to determine whether the relative proportions of temporal scale categories differed between the Atlantic and Gulf lineages. Additionally, we measured total head length (posterior-most point of 8^{th} supralabial to anterior tip of rostral; N = 111), head height (only for photos in lateral aspect; at level of anterior-most point of parietal suture; N = 35), and length of the dorsal posterior temporal (intersection of ventral posterior temporal, dorsal posterior temporal, and adjacent first dorsal scale to intersection of ventral posterior temporal, dorsal posterior temporal, and adjacent ventral temporal; N = 111). All distances were measured using Adobe Photoshop 6.0 with reference to the photographed ruler. We used an analysis of covariance (ANCOVA) to test whether the linear relationship between head length and head height differed between Atlantic and Gulf lineages. We divided the length of the dorsal posterior temporal by head length to control for effects of body size and used an analysis of variance to test whether temporal length differed among the four categories of temporal scales.

We also examined 11 preserved specimens in the Auburn University Museum (AUM) collections. These snakes were from southeastern Georgia and presumed to belong to the Atlantic lineage. For these specimens, we measured length and width of the 6th and 7th infralabial scales with dial calipers. We measured both scales because it was not clear which of these was measured by Krysko *et al.* (2016b) and because they represent the position of the 7th lower labial scale if the mental scale was included and if it was excluded. Additionally, we used photos of the type specimens presented in Krysko *et al.* (2016) to determine length and width of the 6th and 7th infralabial scales using Adobe Photoshop 6.0. A length-to-width ratio was then calculated for each specimen. Mean differences between 6th and 7th infralabials were tested as a paired t-test. Differences between our sample of Atlantic lineage snakes and the type specimens was determined by visual inspection. We used SAS v.9.4 for all analyses (SAS Institute, Inc., 2008) with $\alpha = 0.05$.

RESULTS

GENE SEQUENCE ANALYSIS

We found that the nuclear locus NT3 was completely invariant across all *D. couperi* specimens, and thus NT3 had no variable sites for phylogenetic inference of *D. couperi*. As expected, the inferred ML phylogeny estimated a polytomy (Fig. 2), indicating a lack of phylogenetic structure among individuals from the two mitochondrial lineages of *D. couperi* (*sensu lato*) (Krysko *et al.*, 2016a). Specimens of *D. melanurus erebennus* and *D. melanurus rubidus* clustered as sister taxa to the exclusion of all *D. couperi* specimens when rooted by samples from *Coluber*.

MICROSATELLITE ANALYSIS

We found evidence for null alleles in three loci (Dry33, Dry63, and Dry69). Therefore, we eliminated these loci from further analysis and retained the remaining 14 loci. Delta K was maximized at K = 2 (Fig. 3) and supported two genetic clusters: one associated with the northern-most samples, and a second with the southern-most samples. However, there was extensive admixture between the two clusters, particularly in central Florida (Fig. 4; Fig. 5). For the 20 samples from Krysko *et al.*, (2016a), including representatives from Atlantic and Gulf lineages, we found all individuals were assigned to our southern cluster, and all were highly admixed with the northern cluster (0.66 assignment to the southern cluster for the Gulf clade and 0.57 assignment to southern cluster for the Atlantic clade). Raw likelihood values from Structure began to plateau at K = 3 (Fig. 3) and therefore supported three populations: a cluster of the southern samples, but described further population subdivision of the northern samples into two clusters.

Genetic distance was strongly correlated with geographic distance across the entire range of samples (Mantel r = 0.39; P < 0.001). When our samples were grouped into the Gulf and Atlantic lineages of Krysko *et al.* (2016a), similar effects of isolation by distance were evident within each lineage (Mantel's r = 0.39, p < 0.001 for the Gulf lineage; Mantel's r = 0.40, p < 0.001 for the Atlantic lineage), suggesting no strong barriers to gene flow across the range of *D. couperi* (*sensu lato*).

MORPHOLOGICAL ANALYSIS

Our sample of OCIC specimens recovered four categories of temporal scales from both Atlantic and Gulf lineages (Table 1). In 22% of specimens, temporals conformed to the 2+2

411 formula that Krysko *et al.* (2016b) described as being invariant (Fig. 1A). We found that 38% of
412 specimens exhibited an extra ventral temporal (Fig. 1B), 26% of specimens had extra dorsal and
413 ventral temporals (Fig. 1C), and 14% of specimens exhibited an extra dorsal temporal (Fig. 1D).
414 The frequency with which these four categories occurred differed between Atlantic and Gulf
415 lineage specimens (Table 1; df = 3; Fisher's Exact P = 0.006), with Atlantic lineage snakes
416 tending to have two dorsal temporals and Gulf lineage snakes tending to have three dorsal
417 temporals. Head shape, based on ANCOVA of head width on head length, did not differ between
418 Atlantic and Gulf lineages in either slope (Fig. 7; F = 0.07; df = 1; P = 0.79) or intercept (F =
419 0.48; df = 1; P = 0.49). Length of the dorsal posterior temporal, expressed as a proportion of
420 head length, differed significantly among temporal categories (F = 18.34; df = 3; P < .0001),
421 with the dorsal posterior temporal being proportionately shorter when three dorsal temporal
422 scales are present relative to when two dorsal temporal scales are present (Fig. 6).
423 When the length and width of the 6th and 7th infralabial scales were converted to a length-
424 to-width ratio, the distribution of our sample of scales from Atlantic lineage snakes encompassed
425 values for both type specimens for each scale (Fig. 8). Length-to-width ratios differed between
426 6th and 7th infralabials (t = 8.07; df = 12, P < .0001), with 7th infralabials being more elongate
427 than 6th infralabials.
428

429 # DISCUSSION

430 ## GENE SEQUENCE ANALYSIS

431 We found no variation of the nuclear NT3 locus in sequences generated by Krysko *et al.*
432 (2016a). These authors included this locus because it "potentially represents an informative,
433 single-copy, unlinked locus that is likely evolving at a different rate than mtDNA

(mitochondrial) genes". From this we infer that the authors expected this nuclear gene to corroborate patterns generated from mtDNA, solidifying their conclusion that two species are present. However, if the locus was expected to be informative (i.e., *sensu* Ruane *et al.,* 2014), then the fact that it is invariant indicates that it (1) does not support population structure consistent with two genetic populations (i.e., two species), (2) was not necessary to include it in the study, and (3) only served to restrict phylogenetic inference to the mitochondrial genome and its inherent problems. From a statistical perspective, the only effect of including NT3 in their analysis was a slight reduction of branch lengths due to the sequence's invariance. For these reasons, we suggest that the gene sequence data do not provide compelling evidence in support of two distinct species of *D. couperi* (*sensu lato*).

MICROSATELLITE ANALYSIS

Our population genetic analyses supported two populations within Eastern Indigo Snakes, as did that of Krysko *et al.* (2016a). However, there are two key differences between the population structure described by our microsatellite analysis and their phylogenetic results. First, the geographic pattern of our population clusters suggests a north-south orientation rather than a Gulf-Atlantic orientation. Second, and most importantly, our analyses document widespread contemporary admixture of alleles from the northern and southern populations. Admixture occurs across the entire range of *D. couperi* (*sensu lato*), and cannot be characterized as a narrow hybrid zone. Instead, genetic structure among populations is best described as continuous isolation by distance rather than discrete evolutionary lineages. Meirmans (2012) demonstrated through stimulations that it is possible to have a significant isolation by distance pattern even with two discrete clusters. However, if this was the scenario in our study, we would expect to see

457 a much stronger isolation by distance relationship within lineages relative to across the two
458 lineages recognized by Krysko *et al.* (2016a,b). In fact, the correlation of genetic and geographic
459 distance is nearly identical within and between clusters, which is strong evidence that there are
460 not two separate evolutionary groups of Eastern Indigo Snakes. Based on examination of
461 microsatellites and issues we noted about their migration analysis, we contest the conclusion that
462 the two lineages of Krysko *et al.* (2016a) have "near discrete geographic distributions" (p. 118)
463 and that "dispersal between lineages is too low to influence demographic processes" (p. 119).
464 Rather, these results describe two parapatric populations with high levels of contemporary gene
465 flow, observations that are highly inconsistent with a two-species hypothesis and more in support
466 of a single evolutionary metapopulation lineage species of *D. couperi* (Frankham *et al.*, 2012).
467 Our analyses add to a growing number of examples of Florida organisms that appear,
468 based on modeling of one or few genetic loci, to represent species that are distinct from other
469 mainland counterparts, but for which microsatellite or similar data demonstrate substantial gene
470 flow. Burbrink and Guiher (2014) estimated that there was such low gene flow between Florida
471 Cottonmouths and mainland cottonmouths that speciation must have occurred between the two, a
472 hypothesis immediately contested by data from Strickland *et al.* (2014) who detected a broad
473 geographic range of admixture using AFLP markers. Similarly, Thomas *et al.* (2014) described
474 alligator snapping turtles from the Apalachicola River and adjacent rivers to be a distinct species,
475 despite microsatellite data from Echelle *et al.* (2010) that are inconsistent with this conclusion
476 (Folt & Guyer, 2015). Likewise, Gordon *et al.* (2017) used mtDNA to describe a population of
477 *Anaxyrus boreas* in the western United States as a distinct species, a conclusion contested by
478 microsatellite analysis of gene flow (Forrest *et al.*, 2017).
479

MORPHOLOGICAL ANALYSIS

Contrary to the results presented in Krysko *et al.* (2016b), we reject the hypothesis that the Atlantic and Gulf lineages are identifiable entities revealed by morphology. We reach this conclusion after re-examining the variables used by Krysko *et al.* (2016b) to diagnose each lineage. Of the disparities that emerge between our analyses and theirs, the conformation of the infralabials is the most problematic. The figures presented by Krysko *et al.* (2016b) for the 6th infralabial show great promise for diagnosing lineages, and separation of the lineages along PC1 of their analysis seems to provide statistical support for this character. However, we were struck by how dissimilar Atlantic specimens appeared to be from the scale shape ascribed to them by Krysko *et al*. (2016b). Our analyses demonstrate that the 6th and 7th infralabials differ in shape, that the shape of the 7th infralabial conforms to the shape ascribed to the Atlantic lineage, and that the shape of the 6th infralabial conforms to that ascribed to the Gulf lineage. It is unclear which of these scales was measured by Krysko *et al.* (2016b) and we found that the range of variation of each scale within a sample of Atlantic lineage snakes encompasses both type specimens. One potential explanation for this discrepancy is that Krysko *et al.* (2016b) intended to measure the 7th infralabial but inadvertently measured the 6th for Gulf lineage specimens and the 7th for Atlantic lineage specimens, perhaps because the mental scale sometimes was included in counts and other times was not. Otherwise, we are left with a PCA that separates lineages based on one of the two possible scales, but a univariate analysis that fails to confirm these lineages.

Our results for the temporal scale reveal great variation in the number of these scales present in Eastern Indigo Snakes. The four categories that characterize this variation are found in both Atlantic and Gulf lineage snakes, indicating that this feature is not diagnostic. Nevertheless,

Atlantic lineage snakes tend to have two dorsal temporals, while Gulf lineage snakes tend to have three. We assume that Krysko *et al.* (2016b) intended to measure the dorsal posterior temporal and, therefore, we focused our attention on this scale. Our data indicate that the length of the dorsal posterior temporal, relative to head length, becomes shortened if three dorsal temporals are present and becomes elongate if two dorsal temporals are present. This finding indicates that the scale shapes revealed by PC2 of Krysko *et al.* (2016b) represent distinguishable groups, but these represent two phenotypes and not two species. We infer that the different morphologies of the dorsal posterior temporal result because, during embryonic development of some individuals, the dorsal anterior temporal divides, limiting space for development of the dorsal posterior temporal.

Krysko *et al.* (2016b) also used head shape to diagnose the two lineages. Relative head length and head height did load heavily on their PC1 axis and they used this to diagnose the Atlantic lineage as having an elongate wide head and to diagnose the Gulf lineage as having a short narrow head. Our bivariate examination of head length and height revealed no difference in head shape between the two lineages. We have no convenient explanation for why our results differed from Krysko *et al.* (2016b), although we note that snake morphology can be difficult to measure consistently (Madsen & Shine, 2001). Specimens preserved with mouths open are likely to have larger values for head height than those with mouths closed. If the relative frequency of open-mouthed versus closed-mouthed specimens differed between lineages, this might yield a spurious association of head shape with lineage. Our measurements were made from live specimens with closed mouths, which we infer reduces measurement error. If the lineages truly differ in head shape, our ANCOVA should have revealed this difference.

NATURAL HISTORY MECHANISMS

Our examination of genetic and morphological variation in *D. couperi* (*sensu lato*) demonstrates that the two-species hypothesis proposed by Krysko *et al.* (2016b) is not supported by available data. We offer several explanations for why true patterns of gene flow might result in a lack of genetic and morphological differentiation. First, movements of *D. couperi* can be extensive, especially for males. Male annual home range size is as large as ca. 1500 ha (Hyslop *et al.,* 2014) and average ca. 2.5–6.6 times larger than for females (Breininger *et al.,* 2011; Hyslop *et al.,* 2014). In fact, the disparity between male and female home range sizes becomes exacerbated in large snakes, a feature dominated by data from *D. couperi* (Fig. 9; Appendix III). Within peninsular Florida, male *D. couperi* can move up to ca. 2 km in a single day and average movement distance in males is ca. twice that of females (Bauder *et al.,* 2016a; D. Breininger, unpublished data). Furthermore, males within peninsular Florida increase their movement frequency, distance, and home range size during the breeding season (Bauder *et al.,* 2016a, b). Dispersal distance of males may be 10 times that of females (Stiles, 2013), with a small adult male in southern Georgia dispersing at least 22.2 km (straight line) over approximately two years (Stevenson & Hyslop, 2010).

A second feature of *D. couperi* life history that reduces opportunity for speciation is the variety of habitats used by these animals throughout a year (e.g., Hyslop *et al.,* 2014), particularly in peninsular Florida, where individuals will even utilize habitats with varying degrees of anthropogenic disturbance (Bauder *et al.,* in press). This broad habitat use reduces the opportunity for ecological barriers to gene flow and is consistent with the high rates of gene flow indicated by our microsatellite analysis. Additional life history observations show that *D. couperi* (*sensu lato*) can cross fresh and saltwater features 6–264 m wide (O'Bryan, 2017; D. Stevenson,

549 personal observation; D. Breininger, unpublished data), and we note that traditional river barriers
550 (Soltis *et al.,* 2006) do not appear to limit gene flow in these large snakes. While we do not doubt
551 that a historical climatic event may have separated *D. couperi* into two populations (Krysko *et*
552 *al.*, 2016a), the observed levels of contemporary gene flow indicate that genetic populations of
553 *D. couperi* are in the process of merging back into a single evolutionary population (i.e.,
554 species), as has been observed for diverse taxa following climatic cycles (Frankham *et al.*, 2011).
555 Together, our knowledge of *D. couperi* movement patterns and life history allows us to
556 hypothesize that limited female movement drives structure of the maternally-inherited mtDNA
557 data presented by Krysko *et al.* (2016a), even though high levels of male movement drive
558 extensive gene flow of the nuclear genome (Thorpe *et al.*, 2008). This life history-based model
559 of intersexual variance in *D. couperi* gene flow is consistent with two other reptile systems for
560 which life-history strategies generate contrasting patterns of gene flow among populations. First,
561 female philopatry of Loggerhead Sea Turtles (*Caretta caretta* Linnaeus 1758) causes structuring
562 of mtDNA in the Atlantic Ocean, but high male dispersal drives significant nuclear gene flow
563 among populations (Bowen *et al.,* 2005). Second, a recent study of Neotropical snakes found
564 active-foraging species to have greater rates of nuclear gene flow than ambush-predator species
565 with more limited dispersal (de Fraga *et al.,* 2017). Last, and most relevant, many squamates are
566 polygynous and are characterized by greater male dispersal relative to females (Leturque &
567 Rousset, 2004; e.g., Keog, Webb & Shine, 2007; Dubey *et al.*, 2008; Calsbeek, 2009); therefore,
568 squamates typically have greater nuclear gene flow than that of the mitochondrion (Thorpe *et al.*,
569 2008). This pattern is also seen in many waterfowl species (Scribner *et al.* 2001; Peters *et al.*
570 2012). Thus, we suggest that *D. couperi* is similar to other reptile species in that life history
571 provides explanations for why phylogeographic patterns from mtDNA are inconsistent with

572 historical and/or contemporary patterns of nuclear DNA. Because of this incongruence and the
573 high levels of recent gene flow among populations observed by our microsatellite analysis, we
574 place *Drymarchon kolpobasileus* into synonomy with *D. couperi*. For now, we retain all Eastern
575 Indigo Snakes within *D. couperi*, but note that the analysis of Krysko *et al.* (2016b) reveals that
576 this species may not be diagnosable from *D. melanurus erebennus* (Cope 1960).
577
578 ## CONSERVATION IMPLICATIONS
579 Eastern Indigo Snakes are now being released into the Conecuh National Forest (CNF) of
580 south-central Alabama and to the Apalachicola Bluffs and Ravines Reserve in the panhandle of
581 Florida. Efforts to repatriate the species are the result of extensive collaborations and
582 partnerships between state and federal agencies, as well as non-governmental conservation
583 organizations. Initially, snakes used for release were to come from wild-caught gravid females
584 taken from sites in southeastern Georgia, retained in captivity until they laid eggs, and then
585 released at the point of capture. Eggs from these initial females then were to be hatched in
586 captivity and raised for release at one of the two repatriation sites or donated to the Orianne
587 Center for Indigo Conservation (OCIC), where captive breeding from these and other donated
588 animals was to occur, eventually replacing use of wild-captured females. Many meetings were
589 held among stakeholders and, as Krysko *et al.* (2016a) note, their preliminary molecular results
590 (Krysko, Smith & Smith, 2010) were presented at one of these early meetings as a challenge to
591 the release of Atlantic lineage snakes at the two release sites. Here we describe why data from
592 that preliminary report did not compel changes in the repatriation plan and why continuation of
593 that plan is unlikely to encounter the problems predicted by Krysko *et al.* (2016a).

A lack of apparently viable populations of Eastern Indigo Snakes in the Florida panhandle and southern Alabama and Mississippi was recognized as a significant gap in efforts to retain Eastern Indigo Snakes across its historic geographic range. This gap guided the choice of the two sites where repatriation was deemed reasonable (Enge *et al.,* 2013). Data from Krysko *et al.* (2010) were used to propose that only snakes of the Gulf lineage be used for repatriation because genetic information from two isolated samples from the panhandle of Florida were of this lineage. However, because Eastern Indigo Snakes were extirpated at both sites, discussions centered on how to deal with the uncertainty of which lineage historically occurred at each site and, therefore, which lineage(s) were appropriate to release at each site. This uncertainty recognized the fact that, for both sites, the 3rd and 4th closest localities currently occupied by Eastern Indigo Snakes have the Atlantic lineage. Additionally, the Atlantic lineage occurs in the Suwannee drainage, which empties into the Gulf of Mexico, further illustrating the uncertainty in identifying the location of the Gulf-Atlantic split and that both lineages are found in north Florida where they might serve as colonists for the release sites. Participants in these discussions were made aware of then-unpublished microsatellite data demonstrating widespread gene flow between lineages and observations from the zoo and herpetocultural communities documenting interbreeding of the two lineages with no apparent effects of outbreeding depression. While there is currently no evidence of ecological differences between Atlantic and Gulf lineage snakes, snakes from northern populations are known to differ markedly from southern populations in seasonal activity. Specifically, *D. couperi* in the latter region do not appear dependent upon Gopher Tortoise (*Gopherus polyphemus* Daudin 1802) burrows for winter refugia, likely because of milder winter temperatures (Stevenson, Dyer & Willis-Stevenson, 2003; Hyslop, Cooper & Meyers, 2009; Hyslop *et al.*, 2014; Breininger *et al.,* 2011; Bauder *et al.,* 2016a). As a result, use

of snakes from similar latitudes appeared to be more important in ensuring successful repatriation than use of snakes from the same mitochondrial lineage. The majority of meeting participants therefore concluded that the wide movements of male *D. couperi* and broad habitat use of the species made matching of mitochondrial DNA lineages less important in guiding repatriation than matching spatial variation in ecology. Preservation of mitochondrial lineages was already achieved through apparently viable populations at the northern and southern geographic extremes of *D. couperi* and release of snakes of one mitochondrial lineage into an area historically occupied by the other lineage was viewed simply as replicating patterns of gene flow observed throughout much of north and central Florida.

Those charged with implementing conservation planning for the Eastern Indigo Snake welcome the opportunity to base those plans on the best published science. Because inferences from phylogenetic analyses of few loci can conflict with those from contemporary gene flow, we suggest that, at a minimum, both tools should be considered to adequately reveal recently diverged taxa. Additionally, we argue that a more-consistent voice from the community of systematists will emerge when life-history data are allowed to inform speciation models. For example, the potential for large snakes to move long distances as well as a difference in movement behavior between the sexes limit the utility of mitochondrial data alone to reveal novel species. We suspect this disparity in movement is rampant within squamates (see e.g. Perry & Garland, 2002). Finally, species descriptions for which diagnoses merely report measures of variables that overlap widely among taxa (e.g., Thomas *et al.,* 2014; Krysko *et al.,* 2016b) or that are not derived from results presented in the paper (e.g., Burbrink & Guiher, 2014) should be rejected because they fail to provide further evidence that the proposed taxon is identifiable as an individual. We point to Warwick *et al.* (2015), Sovic *et al.* (2016), and Forrest

et al. (2017) as examples of how careful melding of phylogenetic and population genetic analyses can improve the process by which one might determine whether several populations have diverged sufficiently to be recognized as distinct species. Thus, we urge authors and reviewers to be particularly critical of species descriptions without careful analysis of contemporary gene flow, because these papers can incorrectly delimit species, contribute to erroneous hyperdiversity, and confuse efforts to understand and conserve imperiled biodiversity.

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Whitaker PB, Shine R. 2003. A radiotelemtric study of movements and shelter-site selection by free-ranging brownsnakes (*Pseudonaja textilis*, Elapidae). *Herpetological Monographs* 17: 130–144.

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

936 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

937

938 Appendix I. GenBank accession numbers for sequence data from the nuclear gene *neurotrophin-*

939 3 (NT3) from Krysko *et al.* (2016a) that were analyzed to generate Fig. 2.

Accession number	Species	Country	State	County	Mitochondrial lineage
KT214324	*Drymarchon couperi*	U.S.A.	Florida	Charlotte	Gulf
KT214325	*Drymarchon couperi*	U.S.A.	Florida	Okaloosa	Gulf
KT214326	*Drymarchon couperi*	U.S.A.	Florida	Lee	Gulf
KT214327	*Drymarchon couperi*	U.S.A.	Florida	Citrus	Gulf
KT214328	*Drymarchon couperi*	U.S.A.	Florida	Hendry	Gulf
KT447213	*Drymarchon couperi*	U.S.A.	Florida	Monroe	Gulf
KT214329	*Drymarchon couperi*	U.S.A.	Florida	Miami-Dade	Gulf
KT214330	*Drymarchon couperi*	U.S.A.	Florida	Miami-Dade	Gulf
KT214331	*Drymarchon couperi*	U.S.A.	Florida	Miami-Dade	Gulf
KT214332	*Drymarchon couperi*	U.S.A.	Florida	Monroe	Gulf
KT214333	*Drymarchon couperi*	U.S.A.	Georgia	Coffee	Atlantic
KT214334	*Drymarchon couperi*	U.S.A.	Georgia	Liberty	Atlantic
KT214335	*Drymarchon couperi*	U.S.A.	Georgia	Wayne	Atlantic
KT214336	*Drymarchon couperi*	U.S.A.	Georgia	Bryan	Atlantic
KT214337	*Drymarchon couperi*	U.S.A.	Georgia	Camden	Atlantic
KT214338	*Drymarchon couperi*	U.S.A.	Georgia	Coffee	Atlantic
KT214339	*Drymarchon couperi*	U.S.A.	Georgia	Telfair	Atlantic
KT214340	*Drymarchon couperi*	U.S.A.	Georgia	Evans	Atlantic
KT214341	*Drymarchon couperi*	U.S.A.	Georgia	Irwin	Atlantic
KT447212	*Drymarchon couperi*	U.S.A.	Georgia	Wheeler	Atlantic
KT214342	*Drymarchon couperi*	U.S.A.	Georgia	Liberty	Atlantic
KT214343	*Drymarchon couperi*	U.S.A.	Florida	Brevard	Atlantic
KT214344	*Drymarchon couperi*	U.S.A.	Florida	Brevard	Atlantic
KT447212	*Drymarchon melanurus erebennus*	U.S.A.	Texas	Hidalgo	-
KT447213	*Drymarchon melanurus rubidus*	Mexico	Sonora	-	-
KT447214	*Coluber flagellum*	U.S.A.	Florida	Washington	-
KT447215	*Coluber constrictor*	U.S.A.	Florida	Highlands	-

940

941 Appendix II. Multiplex PCR panels for *Drymarchon couperi* microsatellite loci. The names of

942 loci are as in Shamblin *et al.* (2011).

Multiplex	Locus
1	Dry24 – 6FAM
	Dry55 – 6FAM
	Dry30 – VIC
	Dry44 – PET
	Dry68 - NED
2	Dry48 – 6FAM
	Dry63 – 6FAM
	Dry58 – VIC
	Dry59 – VIC
	Dry65 – VIC
	Dry69 - VIC
	Dry06 – PET
	Dry33 - NED
3	Dry05 – 6FAM
	Dry35 – VIC
	Dry14 – PET
	Dry70 – NED

943

944 Appendix III. Citations for snake studies (N = 28) describing intersexual variance in home range
945 size (hectares) for 22 species of snakes used to generate Fig. 9. See Macartney *et al.* (1988) for
946 an earlier review of the topic.

Species	Home range size (ha)		Citations
	Male	Female	
Agkistrodon contortrix	9.9	3.4	Fitch (1960)
Agkistrodon piscivorus	1.86	0.31	Roth (2005)
Bothrops asper	6.3	5.8	Wasko and Sasa (2009)
Carphophis amoenus	0.028	0.004	Clark (1970)
Coluber constrictor	9.3	3.9	Fitch (1958)
C. constrictor	3	1.8	Fitch (1963b)
Coluber flagellum	150	21	Dodd and Barichivich (2007)
Coronella austriaca	0.66	0.45	Goddard (1980)
Crotalus horridus	143.8	19.9	Rudolph and Burgdof (1997)
Crotalus viridis	12.1	6.5	Fitch (1949)
Drymarchon couperi	510	101	Hyslop *et al.* (2014)
D. couperi	202	76	Breininger *et al.* (2011)
D. couperi	141	22	Moler (1985)
D. couperi	74	19	Layne and Steiner (1996)
D. couperi	149	49	Bauder *et al.* (2016a)
Hoplocephalus bungaroides	2.2	2.1	Webb and Shine (1997)
Hoplocephalus stephensii	20.2	5.4	Fitzgerald *et al.* (2002)
Lampropeltis getula	49.5	49.4	Linehan *et al.* (2010)
Morelia spilota	12	12	Shine and Fitzgerald (1996)
Nerodia sipedon	0.51	0.58	Roth and Greene (2006)
Pantherophis obsoletus	11.7	9.3	Fitch (1963a)
P. obsoletus	9.5	9.5	Durner and Gates (1993)
Pituophis catenifer	36.6	26.9	Kapfer *et al.* (2010)
Pituophis melanoleucus	69	43	Gerald *et al.* (2006)
P. melanoleucus	70.1	37.5	Miller *et al.* (2012)
Pseudonaja textilis	11.8	1.5	Whitaker and Shine (2003)
Sistrurus catenatus	27.8	21.7	Johnson (2000)
Thamnophis sirtalis	14.2	9.2	Fitch (1965)
Vipera aspera	0.29	0.18	Naulleau (1968)

947

Fig. 1. Head scale patterns in Eastern Indigo Snakes (*Drymarchon couperi*). A) 2+2 condition of temporals (I = dorsal anterior temporal; II = ventral anterior temporal; III = dorsal posterior temporal; IV = ventral posterior temporal) and position of 4^{th}, 5^{th}, 6^{th}, and 7^{th} infralabials; B) 3_v+2 condition of temporals (extra ventral temporal shaded); C) 4+2 condition of temporals (extra dorsal and ventral temporals shaded); D) 3_d+2 condition of temporals (extra dorsal temporal shaded).

Fig. 2. Maximum likelihood phylogeny among samples of Eastern Indigo Snakes (*Drymarchon couperi, sensu lato*) and outgroups (*Drymarchon melanurus*, *Coluber constrictor*, *Coluber flagellum*), as inferred from sequence data from the nuclear gene neurotrophin-3 (NT3). Indigo snakes are labeled by GenBank accession numbers and are classified by the lineages identified by Krysko *et al.* (2016a; blue = Atlantic lineage; orange = Gulf lineage).

Fig. 3. Plot of Delta K (A) and likelihood scores (B) used to identify the most likely number of population clusters across the range of *Drymarchon couperi* using the Bayesian algorithm Structure (Evanno, Regnaut & Goudet, 2005). The dashed line in (A) indicates Delta K = 0; the error bars in (B) indicate S.D.

Fig. 4. Bar plots of population clustering estimated through the Bayesian clustering algorithm Structure with (A) K = 2 and (B) K = 3. The y-axis is the proportion of individual ancestry for each cluster; in the x-axis, group 1 represents individuals assigned or assumed to be within the Atlantic mitochondrial clade, and group 2 represents individuals assigned or assumed to be in the Gulf mitochondrial clade. Within each group, individuals are sorted by latitude. (A) K = 2; red indicates alleles from the northern population cluster, and green indicates alleles from the southern population cluster. (B) K = 3; green and blue indicate alleles from two northern population clusters, and red indicates alleles from the southern population cluster.

971 **Fig. 5.** Maps of sampling sites represented as pie charts of percent ancestry within population
972 clusters identified by Structure analyses. (A) K = 2 populations, with the southern cluster
973 represented as black and the northern cluster as gray; (B) K = 3 populations, with the southern
974 cluster as black and two northern populations as grey and white. Lines indicate state boundaries
975 in the southeastern U.S.A.

976 **Fig. 6.** Bivariate plot of head height on head length. Values from Atlantic lineage indicated by
977 solid circles and solid line; values from Gulf lineage indicated by open circles and dashed line.

978 **Fig. 7.** Box and whiskers plot of distribution of ratio of dorsal posterior temporal scale length to
979 head length in four categories of temporal scales (see Fig. 1). Vertical lines indicate range; box
980 indicates interquartile, horizontal line indicates median; open diamond indicates mean.

981 **Fig. 8.** Distribution of length-to-width ratio of 6^{th} and 7^{th} infralabials in 11 Atlantic lineage
982 specimens of *Drymarchon couperi* (dark spots). Open triangles indicate ratios from type
983 specimen of *D. couperi* (Atlantic lineage); open diamonds indicate ratios from type specimen of
984 *D. kolpobasileus* (Gulf lineage).

985 **Fig. 9.** Bivariate plot of female home range area (ha) on male home range area for 25 studies of
986 21 species of snakes (Appendix III). Open circles are data from *Drymarchon couperi*; closed
987 circles are data from all other species of snakes. Dashed line is null expectation if male and
988 female home range sizes are equal in size.

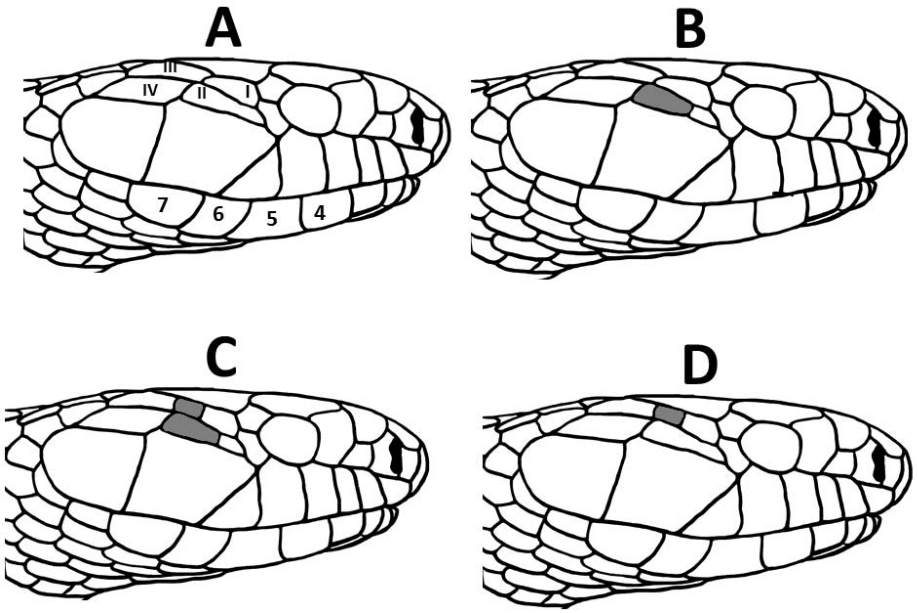

Fig. 1

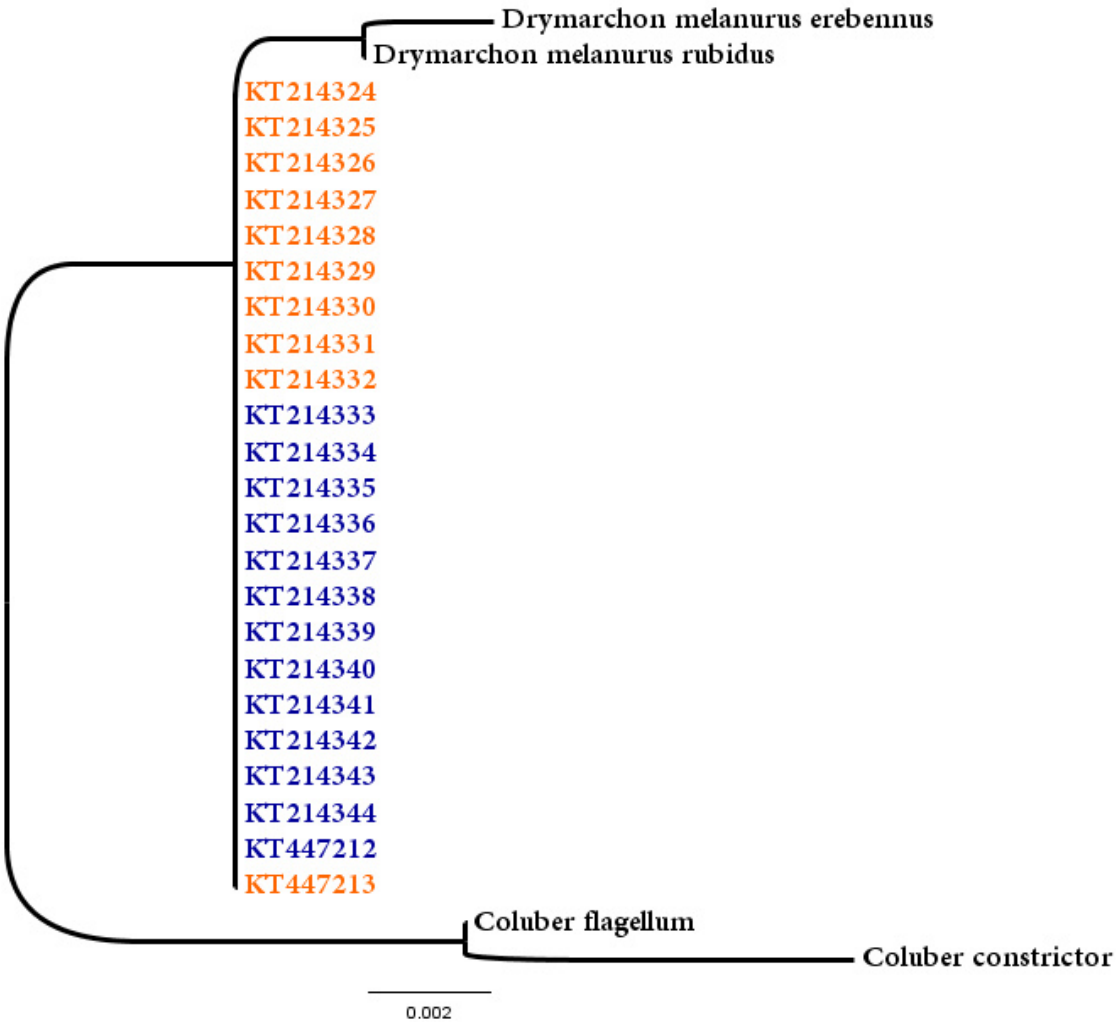

Fig. 2

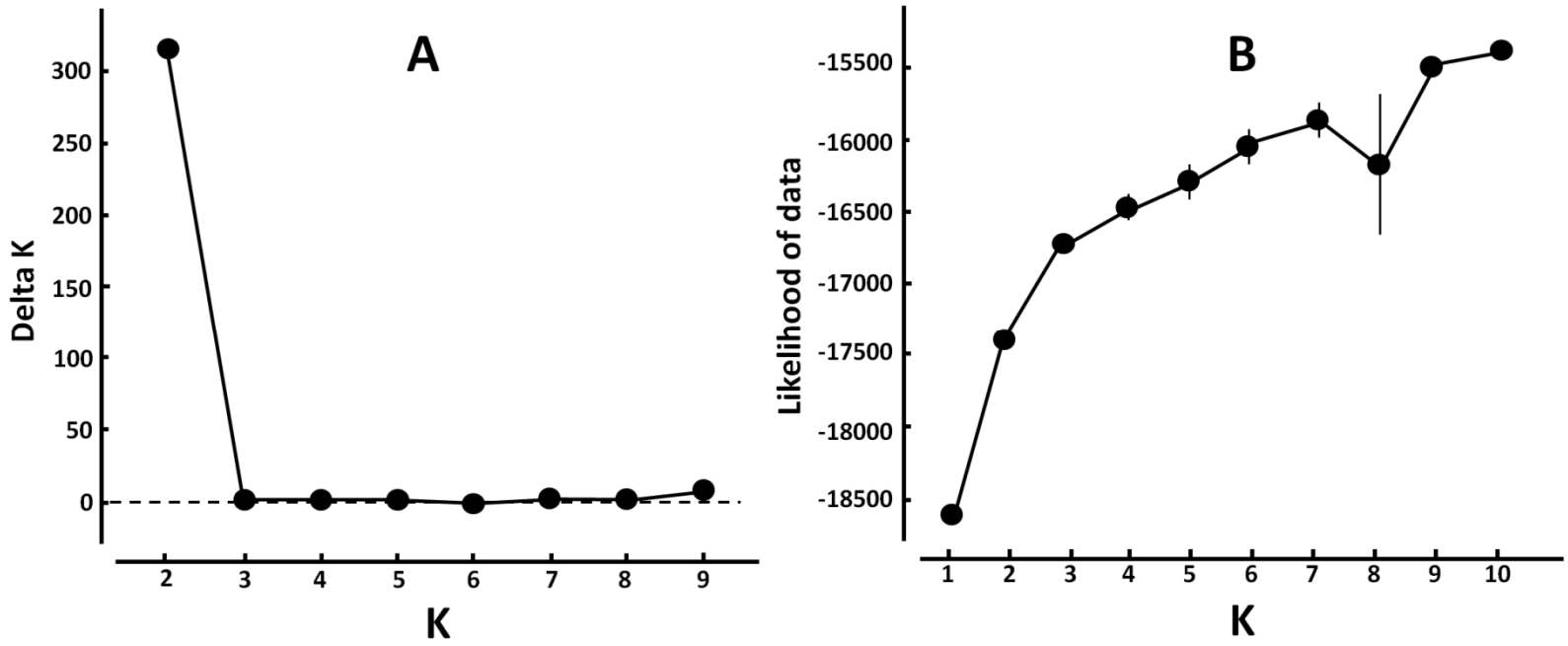

Fig. 3

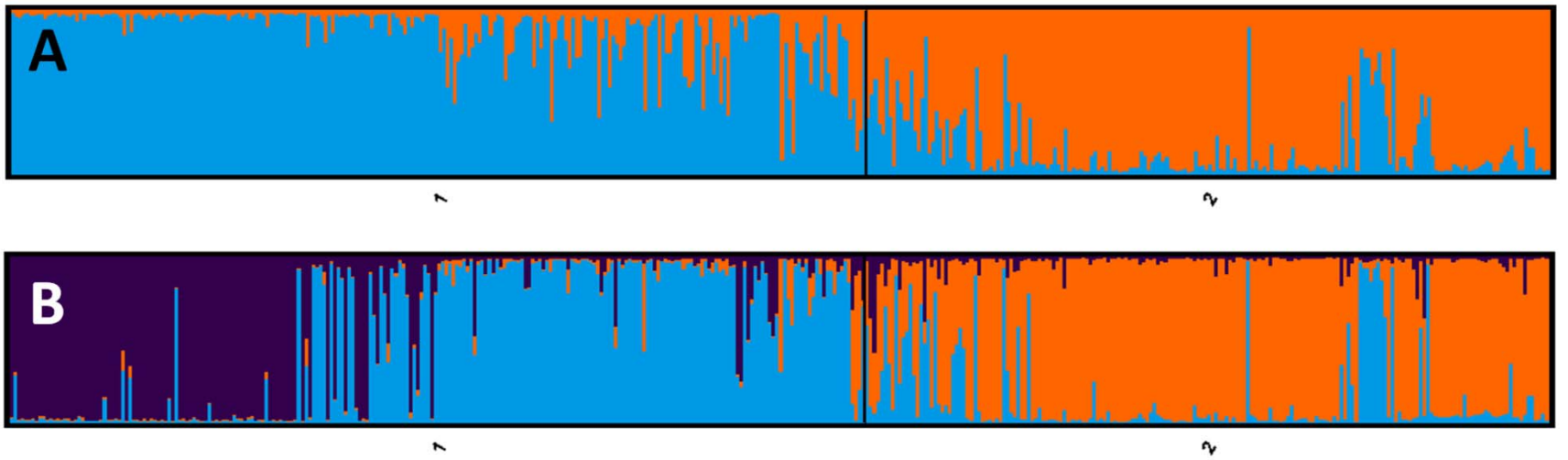

Fig. 4

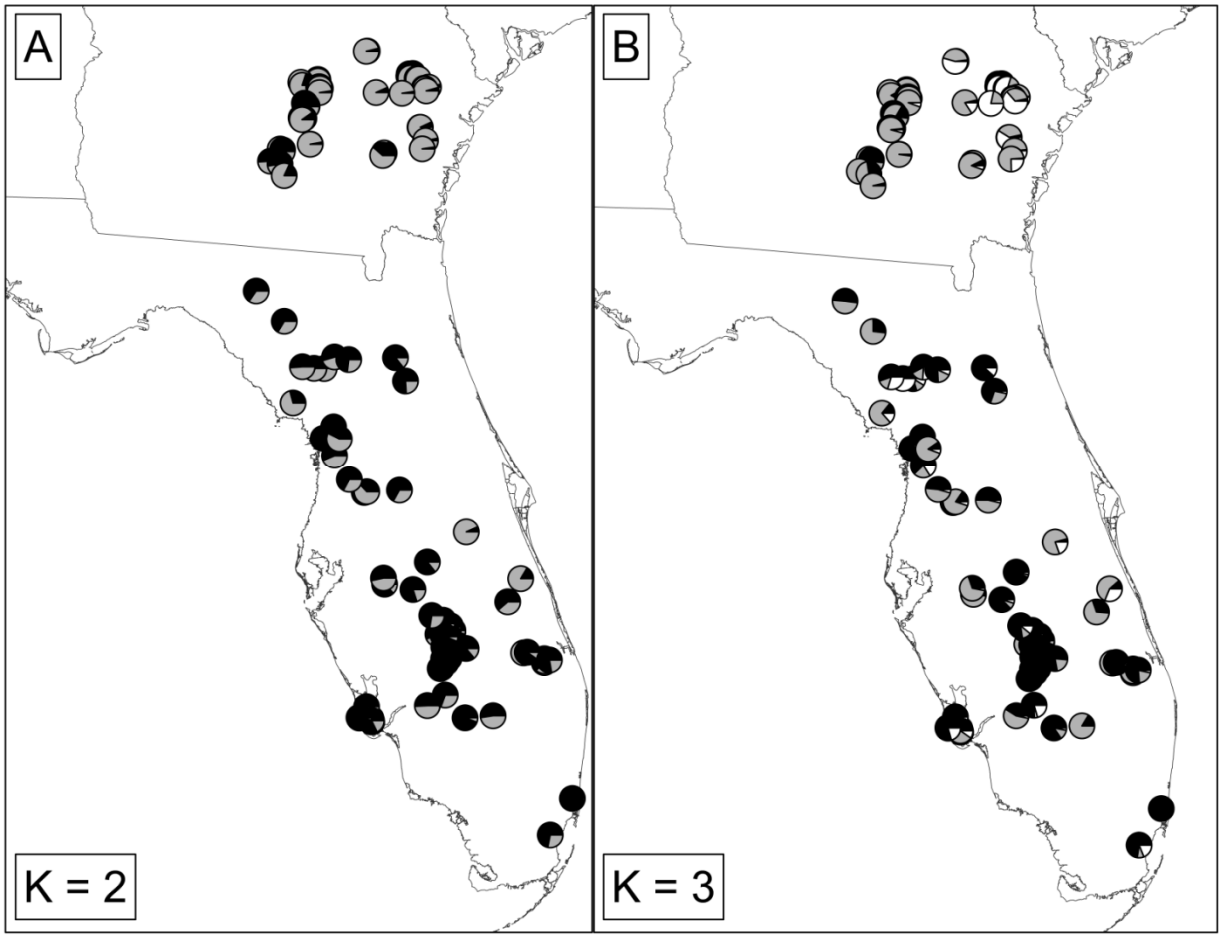

Fig. 5

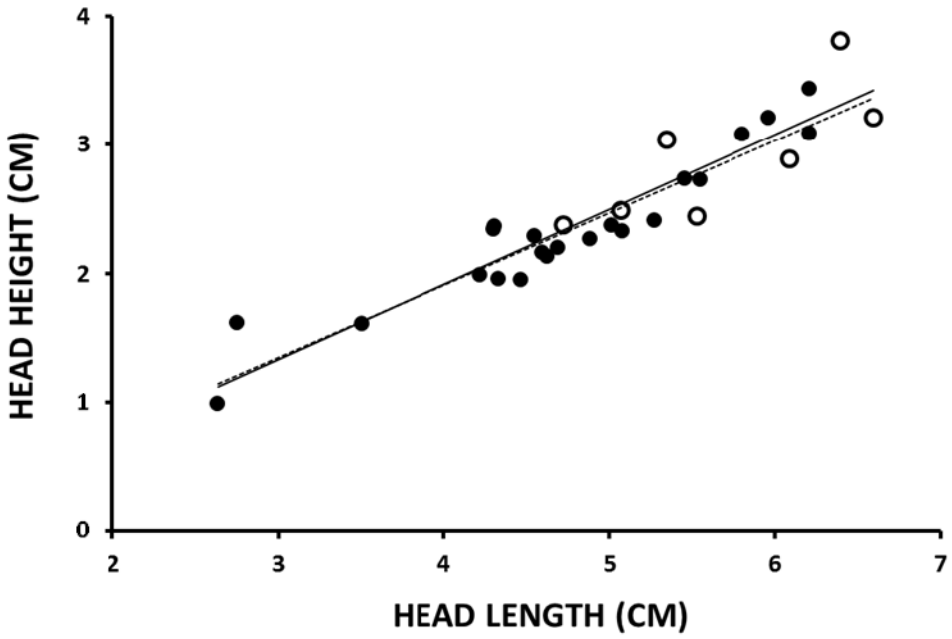

Fig. 6

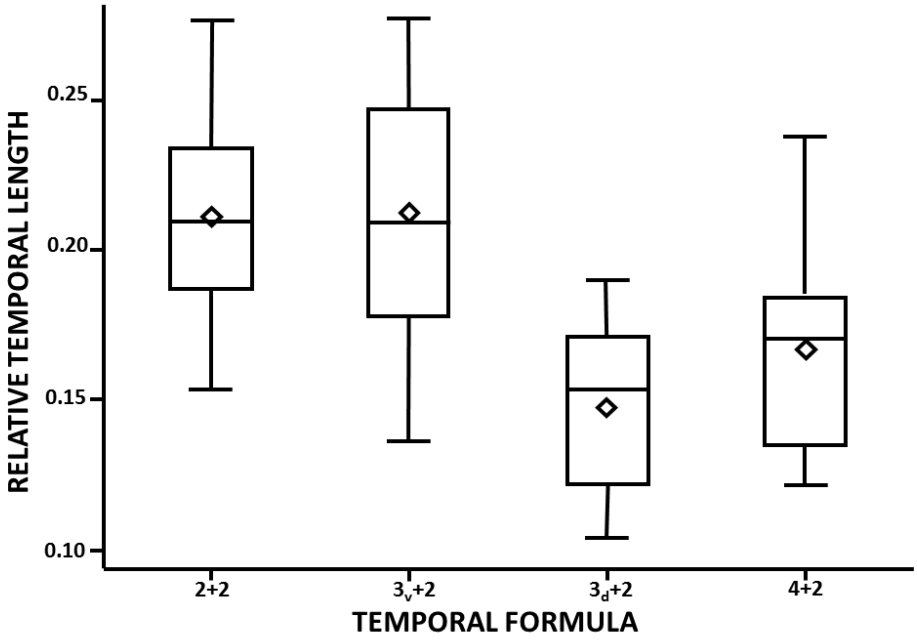

Fig. 7

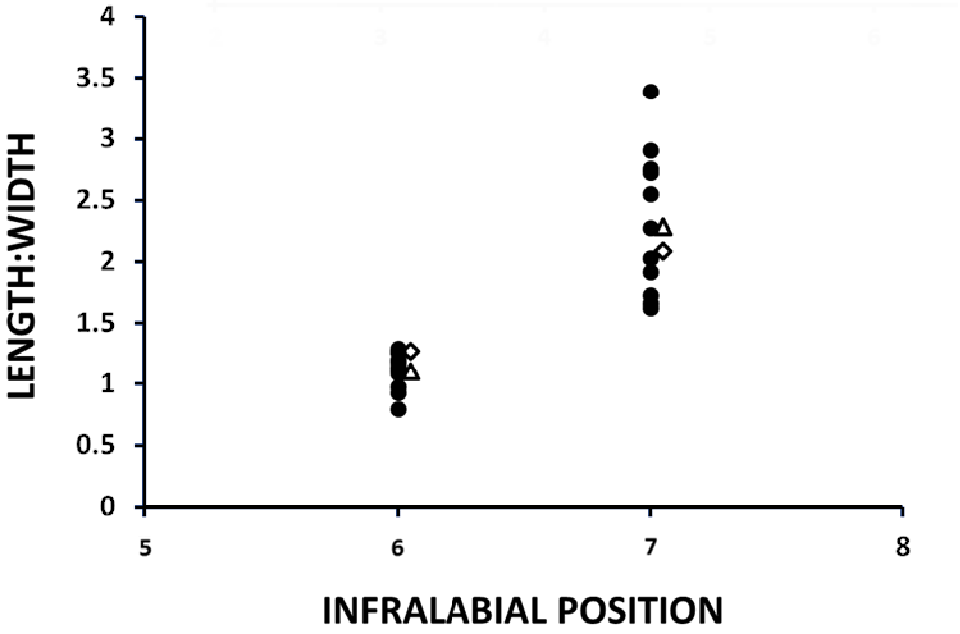

Fig. 8