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Genetic divergence correlates with the contemporary landscape in populations of Slimy Salamander (*Plethodon glutinosus* species complex) across the lower Piedmont and Coastal Plain of the southeastern United States

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Genetic divergence correlates with the contemporary landscape in populations of Slimy Salamander (*Plethodon glutinosus* species complex) across the lower Piedmont and Coastal Plain of the southeastern United States. Walter H. Smith, Jessica A. Wooten, Carlos D. Camp, Dirk J. Stevenson, John B. Jensen, Megan Turner, and N. Reed Alexander

Abstract: A primary goal of landscape genetics is to elucidate factors associated with genetic structure among populations. Among the important patterns identified have been isolation by distance (IBD), isolation by barrier (IBB), and isolation by environment (IBE). We tested hypotheses relating each of these possible patterns to genetic divergence in Slimy Salamanders (*Plethodon glutinosus* (Green, 1818) species complex) across the lower Piedmont and Coastal Plain of Georgia and adjacent areas of South Carolina. We sequenced 2148 total bp, including three regions of the mitochondrial genome and a nuclear intron, and related genetic distance to GIS-derived surrogate variables representing possible IBD (geographic distance), IBE (principal components of 19 climate variables, watershed, and Normalized Difference Vegetation Index or NDVI), and IBB (streams of 4th and higher order). Multiple Matrix Regression with Randomization analysis indicated significant relationships between genetic distance and two principal components of climate, as well as NDVI. These results support roles for environment (IBE) in helping to drive genetic divergence in this group of salamanders. The absence of a significant influence of IBD and IBB was surprising. It is possible that the signal effects of geographic distance and barriers on genetic divergence may have been erased by more recent responses to the environment.

Key Words: amphibian, divergence, landscape, *Plethodon glutinosus*, Slimy Salamander

Introduction

Genetic structure within and among populations is frequently correlated with landscape variables that enable or preclude gene flow (Spear et al. 2005; Marsh et al. 2007). These may include the presence of physical barriers to dispersal, which has long been implicated in inter-population divergence (isolation by barrier, IBB; Palumbi 1994; Murphy et al. 2010). Genetic differentiation may also result from isolation by distance (IBD), in which divergence correlates with geographic distance. This happens when gene flow between local populations is not sufficient to erase the effects of drift within them (Wright 1943; Slatkin 1993; Rousset 1997). More recently, landscape ecologists have recognized the influences of isolation by environment (IBE; Orsini et al. 2013; Wang 2013; Wang and Bradburd 2014), in which population structure and gene flow correlate with the heterogeneity of environmentally diverse landscapes.

Another important aspect of landscape genetics that has recently emerged is that of temporal scale. Because of the lag time of potentially many generations between dispersal and genetic divergence, genetic distance is expected to reflect the importance of historical aspects of the landscape rather than contemporary ones (Manel and Holderegger 2013). However, results of empirical studies indicate that contemporary features of the landscape can be more strongly correlated with genetic distances than historical ones (Zellmer and Knowles 2009; Manel and Holderegger 2013).

Landscape factors can have especially strong effects on genetic divergence within species that are dispersal-limited (Chenoweth et al. 1998; Planes and Fauvelot 2002; Funk et al. 2005; Cunningham et al. 2009). Terrestrial salamanders of the genus *Plethodon* in particular have low dispersal rates (Marsh et al. 2004; Ousterhout and Liebgold 2010), with some species dispersing an average of no more than five meters within a generation (Cabe et al. 2007). Moreover,

members of this genus are often characterized by high levels of genetic divergence and cryptic speciation (Highton 1989, 1997; Carstens et al. 2004; Shepard and Burbrink 2008; Liebgold et al. 2011; Camp and Wooten 2016). Therefore, elucidating the role that landscape variables play in influencing divergence patterns within this genus may help in gaining further insights into the process of speciation. Inter-population divergence within species of *Plethodon* has been related to IBE (Fisher-Reid et al. 2013), as well as to both IBD (Gibbs 1998; Cabe et al. 2007; Shepard and Burbrink 2011) and IBB (Marsh et al. 2007, 2008; Shaidani 2017). Moreover, patterns of genetic divergence can be related to contemporary (Gibbs 1998), as well as historical (Jordan et al. 2009), features of the landscape in this taxonomic group.

Salamanders of the genus *Plethodon* occur throughout the Piedmont and Coastal Plain regions of the southeastern United States, a region characterized by high levels of species richness (Keddy et al. 2006; Murdoch et al. 2007; Buhlmann et al. 2009), endemism (Sorrie and Weakley 2006), and within-species genetic diversity (Bermingham and Avise 1986; Osentoski and Lamb 1995; Donovan et al. 2000; Austin et al. 2002; Beamer and Lamb 2008; Fontanella et al. 2008; Walker et al. 2009). This is an environmentally heterogeneous region that is bisected by potentially significant barriers to gene flow in the form of major rivers (Edwards et al. 2013). Moreover, habitats across this region are often fragmented because of patterns of current and past land use (Turner and Ruscher 1988). Therefore, this is an ideal area for testing hypotheses related to gene flow and genetic divergence in dispersal-limited species.

For our study system, we chose the salamanders of the Slimy Salamander, *Plethodon glutinosus* (Green, 1818) species complex, which occur in forested habitats throughout the area. Originally described as a single, widespread species by Green (1818), this complex was split into 14 parapatric species by Highton (1989) based on clustering geographic populations with similar

allozyme profiles. Six of these currently recognized species occur within the lower Piedmont and Coastal Plain provinces of Georgia alone. These include *P. chlorobryonis* Mittleman, 1951, *P. glutinosus* (Green, 1818), *P. grobmani* Allen and Neill, 1949, *P. ocmulgee* Highton, 1989, *P. savannah* Highton, 1989, and *P. variolatus* (Gilliams, 1818). Our purpose was to test whether patterns of genetic divergence within these species support the hypotheses of IBD, IBB, and/or IBE in this region of Georgia and the adjacent area of South Carolina. We were particularly interested in relationships with measures of the contemporary landscape.

Materials and methods

Study organisms

Slimy Salamanders are terrestrial members of the lungless salamander family, the Plethodontidae, which comprises nearly two-thirds of all known salamander species. The 14 parapatric members of the *P. glutinosus* species complex are residents of deciduous forests from Texas to New England. Under moist conditions they forage for invertebrates on the forest floor, otherwise taking cover under rocks, woody debris, and in underground refugia (Petranka 1998).

We collected a total of 114 specimens from 66 localities, primarily from the lower Piedmont and Coastal Plain of Georgia (Table 1; Fig. 1). We included specimens from throughout the geographic ranges identified by Highton (1989) for *P. ocmulgee* and *P. savannah*. We collected additional specimens from well into the ranges identified by Highton (1989) for neighboring *P. glutinosus*, *P. grobmani*, *P. variolatus*, and *P. chlorobryonis*. For greater context, we collected an additional 14 specimens from eight more distant locations within the respective ranges of those neighboring species (Table 2; Fig. 1).

Salamanders readily regenerate their tails following amputation, including both central and peripheral components of the nervous system (Mchedlishvili et al. 2012). Therefore, in order to avoid destructive sampling, we harvested tissue for genetic analysis by clipping approximately 1 cm of the tip of the tail. Each tail tip was stored in absolute ethanol to await DNA extraction. We then released each specimen at its point of capture.

Molecular methods

We used Qiagen's DNeasy Blood and Tissue kit (Valencia, CA, USA) to isolate DNA from the tail tissue. We amplified three segments (1824 total bp) of the mitochondrial genome — 12S rRNA-valine tRNA (*12S rRNA*), cytochrome b (*cytb*), and NADH dehydrogenase 4 (*ND4*) — using primers published by Titus and Larson (1996) and Mortiz et al. (1992). We also amplified an intron (324 bp) of the nuclear gene interleukin enhancer binding factor 3 (*ILF3*) using primers published by Fisher-Reid and Wiens (2011). PCR amplicons were cleaned up using ExoSapIT (Thermo-Fisher Scientific) following the manufacturer's instructions. We sent all purified PCR products to Molecular Cloning Laboratories (<http://www.mclab.com>) for Sanger sequencing.

We created mitochondrial and nuclear networks of DNA haplotypes to better visualize the relationships among haplotypes using the computer program PopART: Population Analysis with Reticulate Trees (Leigh and Bryant 2015). For the mitochondrial network, the haploid mitochondrial genes were concatenated, and the median joining network option was used to calculate relationships among individuals in the network based on pairwise differences among haplotypes (Bandelt et al. 1999). In order to obtain haplotypes from the diploid nuclear DNA, we used the program seqPHASE (Flot 2010) to prepare our FASTA file for use in the computer

program PHASE: a software package for Phylogenetics and Sequence Evolution (Jow et al. 2002; Hudelot et al. 2003) to predict haplotypes for each individual. The two haplotypes for each individual were used to estimate a nuclear DNA haplotype network using the median joining network in PopART.

We calculated genetic distances between individuals using the measure for individual genetic distance by Smouse and Peakall (1999) in the R package PopGenReport (Adamack and Gruber 2014; Gruber and Adamack 2015). We recognized that our measure of genetic distance was based significantly on three linked regions of the mitochondrial genome. Phylogenetic inferences based on mtDNA often disagree with those based on nDNA (Rubinoff and Holland 2005; Toews and Brelsford 2012), including within the genus *Plethodon* (Fisher-Reid and Wiens 2011). However, because of a smaller effective population size, mitochondrial genes normally fix new mutations at a faster rate than nuclear ones (Ballard and Whitlock, 2004). Consequently, nDNA tends to be a lagging indicator of population divergence (Zink and Barrowclough 2008). Therefore, nuclear genes may be rather poor measures of recent divergences, and this is apparently the case even at the species level in the *P. glutinosus* complex (Fisher-Reid and Wiens 2011). Therefore, we felt that relying on mtDNA for much of our measure of divergence among populations within this complex was justified.

Environmental data collection, coding of populations, and statistical analysis

We used ArcGIS v10.1 (<https://www.arcgis.com/features/index.html>) or DIVA-GIS v7.5 (<http://www.diva-gis.org>) to extract variables associated with individual locations in order to use them in our analyses. To test the hypotheses of IBD, IBB, and IBE, we assigned one or more of these variables (Table 3) as surrogate measures for distance (IBD), barrier (IBB), or environment

(IBE). Geographic distance was straightforward; it was simply the geographic distance between pairwise sampling localities. We used ArcGIS v10.1 to produce a pairwise matrix of geographic distances between populations.

As a surrogate measure for barrier, we used streams of 4th order and higher. Even low-order (1st and 2nd) streams impede gene flow in *Plethodon* (Marsh et al. 2007); therefore, we chose a stream-order level sufficiently high enough to represent a significant barrier to gene flow. The placement of these streams was used to create a binary matrix representing the location of populations with respect to each stream as a putative barrier; that is, each pair of populations was coded with 0 if located on the same side of a putative barrier and with 1 if located on opposite sides of the barrier. This coding scheme was similar to that originally used by Kozak et al. (2006a).

We used three different measures for environment. The first was climate, the second was Normalized Difference Vegetation Index (NDVI), a satellite-generated measure of plant density, and the third was the watersheds of the 4th-order streams we had chosen as barriers. We chose this last measure because divides between watersheds may represent dry areas across which salamander travel can be restricted (Peterman et al. 2014) and because the connectedness of mesic habitats associated with watersheds is correlated with genetic divergence in at least one species of *Plethodon* (Shepard and Burbrink 2008).

We obtained measures of climatic similarity for each sampled population from the WorldClim dataset (worldclim.org) by overlaying the location of each sample collection with 19 BioClim layers representing measures of temperature and precipitation (Table 4) interpolated from approximately 50,000 weather stations worldwide in ArcGIS v10.1 (Hijmans et al. 2005). Measures for each climate variable were extracted at each point location where tissue was

collected using DIVA-GIS (diva-gis.org). Because of the potential for correlation among several of these variables, we used principal-components analysis (PCA) in SPSS (IBM.com) to extract independent measures of climate. We created a pairwise matrix of climatic dissimilarity between populations for each of the important PCs in R (R Core Team 2013). Site-specific values for NDVI were extracted using DIVA-GIS v7.5. We created a pairwise matrix of dissimilarity between populations for each of the variables and NDVI using the 'dist' function with the default Euclidean distance in R (R Core Team 2013). We created a binary matrix representing the location of populations with respect to each watershed similar to that used for streams. Each pair of populations was coded with 1 if they belonged to the same watershed and 0 if they belonged to different watersheds.

Species within this complex have been hypothesized to have diverged in allopatry and to have since come into secondary contact (Highton 1995; Highton et al. 2012). Therefore, to remove the potentially confounding effect of species, we relied on sampling localities reported by Highton (1989) for each of the six species that inhabit the region. We first drew a polygon connecting the outermost collecting localities in Highton (1989) for each species to determine the minimum geographic range occupied by that species. We then assigned all of our sampled populations that fell within each respective polygon to that particular species. For sampling localities that fell between polygons, we drew a line equidistant between adjacent polygons and assigned our sampling localities to the nearest respective species. The one exception to this was for a locality that was closer to *P. savannah* than to *P. cholorobryonis* but which we assigned to the latter species. These specimens came from a bluff in eastern Burke County on the western bank of the Savannah River. Highton (1989) identified populations from northern Burke County (24 km to the northwest) and specimens from west of Burke County (Jefferson County) as *P.*

savannah. Moreover, all of the specimens within this region identified by Highton (1989) as *P. chlorobryonis* occurred on the eastern side of the Savannah. However, we assigned the eastern Burke specimens to *P. chlorobryonis* because they clearly possessed the greenish lateral markings originally described as characteristic of *P. chlorobryonis* (Mittleman 1951). We particularly checked the genetic affinities of these specimens to determine if our assignment was warranted. After assigning all specimens to species, we created a binary matrix representing the location of populations with respect to each species; populations were coded with 1 if they belonged to the same species and with 0 if they did not.

To analyze the possible relationships between the above variables and genetic distance, we developed a dissimilarity matrix for genetic distance between purported populations in R (R Core Team 2013). Next, we ran a correlation analysis to assure that our chosen variables were independent using SPSS (ibm.com). Using the rescale function in R (R Core Team 2013), we scaled each matrix to values between 0 and 1. This numerical transformation was necessary to ensure that our variables were of the same order of magnitude. We then used multiple matrix regression with randomization (MMRR) after Wang (2013) and Nanninga et al. (2014) in the CRAN package PopGenReport (Adamack and Gruber 2014; Gruber and Adamack 2015) in R (R Core Team 2013). This procedure is capable of including any number of variables that are represented by distance matrices and generates an output based on multiple regression. It quantifies the significance of each variable and the fit of the overall model for the given independent and dependent variables (Wang 2013). In our case, the independent variables were geographic distance, streams, NDVI, watersheds, climate PCs, and species. The last variable was included to remove the possibly confounding effect of taxon. Because two analyses (correlation

and MMRR) were run on the same data, we adjusted the alpha level for significance testing from 0.05 to 0.033 using the method to control the false discovery rate described by Narum (2006).

Results

Gene sequences from our sampled populations showed that there were three major clusters of haplotypes as determined by the program Network, based on mutations in unique haplotypes (Fig. 2). These clusters largely corresponded with the taxa groupings *P. chlorobryonis/variolatus*, *P. grobmani*, and *P. ocmulgee/savannah*. Across the taxa groupings, there was a total of 90 unique haplotypes, with an estimated 299 mitochondrial and 18 nuclear mutations in the haplotype networks. There was a particularly high level of genetic distance between the haplotype group representing *P. chlorobryonis/variolatus* and the other groups. Of particular note, haplotypes from the green-sided specimens collected in eastern Burke County grouped within that representing *P. chlorobryonis/variolatus*, indicating that our morphology-based species assignment of those specimens to *P. chlorobryonis* was valid. The haplotypes from populations recognized as *P. glutinosus* did not group together but were scattered among the major clusters.

PCA of the BioClim climate variables yielded four PCs that cumulatively accounted for 93.7 % of the total variance (Table 5). PC1–4 accounted for 58.9 %, 20.1 %, 8.3 %, and 6.3 % of the total variance, respectively. PC1 was driven by measures of temperature mean and range, as well as by variation in precipitation and precipitation during summer. PC2 was most strongly driven by maximum temperature and annual precipitation. PC3 was primarily determined by isothermality. PC4 was related largely to mean temperature during the driest quarter. The

correlation analysis of these four PCs and the other variables indicated no significant correlations between any pair.

The results of MMRR analysis indicated that four variables were significantly associated with genetic distance. These were species, climate PC1, NDVI, and climate PC4 (Table 6). None of the other variables were significantly related to genetic distance (geographic distance, climate PC2, climate PC3, watersheds, streams). The F value for the MMRR-generated model was 180.9376 ($P = 0.001$), with a total r^2 of 0.1470.

Discussion

We found considerable genetic structure in Slimy Salamanders across the lower Piedmont and Coastal Plain of Georgia and adjacent areas. The major clusters of mitochondrial haplotypes were generally associated with recognized species. Mitochondrial haplotypes formed three primary clusters associated with three respective taxonomic groupings: *P. chlorobryonis/variolatus*, *P. grobmani*, and *P. ocmulgee/savannah* (Fig. 2). This pattern explains the strong association between genetic distance and species in our analysis. A few haplotypes did not group with their expected haplotype cluster but with those of the geographically neighboring group. This could have been the result of our misidentification of certain boundary populations, or it could reflect hybridization between parapatric neighbors, a phenomenon which occurs commonly between species of this and related species complexes (Highton and Peabody 2000; Weisrock et al. 2005). There was considerably less diversity of nuclear haplotypes (Fig. 3). This was not unexpected given that nuclear divergence is known to lag behind that of the mitochondrial genome (Ballard and Whitlock 2004).

The greatest divergence occurred between the *P. chlorobryonis/variolatus* group and the two other taxonomic groupings. This deep separation was reflected in both mitochondrial and nuclear haplotypes, while the other mitochondrial split was not as evident. This pattern suggests that *P. chlorobryonis/variolatus* diverged from the others much earlier than *P. grobmani* did from *P. ocmulgee/savannah*. It also suggests that individual species have not fully separated genetically. Genetic similarities between *P. chlorobryonis* and *P. variolatus* and between *P. ocmulgee* and *P. savannah* have been previously noted (Kozak et al. 2006b; Wiens et al. 2006), leading some to suggest that these individual species distinctions may not be valid (e.g., Wiens et al. 2006). The resolution of this problem awaits a broader genetic analysis of the group than we were able to undertake. However, the pattern of divergence in our data conflicts with the assertion by Highton et al. (2012) that all members of this complex speciated concurrently.

In addition to species, climate PCs 1 and 4 were significant contributors to patterns of genetic distance between populations, along with NDVI. This supports the hypothesis that genetic divergence within this group of salamanders is strongly influenced by IBE. While earlier workers have suggested that IBE may be a dominant factor in genetic divergence among populations (Thorpe et al. 2008; Nosil 2012), Wang et al. (2013) argued that geographical factors are more important than ecological ones based on an analysis of *Anolis* lizards. Their results agree with the general view that older features of the landscape better explain genetic divergence than do contemporary ones. In fact, both IBB (Marsh et al. 2007, 2008; Shaidani 2017) and IBD (Gibbs 1998; Cabe et al. 2007; Shepard and Burbrink 2011) are related to divergence in the genus *Plethodon*. In contrast, however, the analysis conducted by Zellmer and Knowles (2009) indicated that genetic divergence in Wood Frogs (*Lithobates sylvaticus* (LeConte, 1825)) reflects the contemporary landscape more so than historic features.

Given the lack of a significant relationship between genetic divergence and either stream barriers or geographic distance, our results agree with the view that contemporary, or at least very recent, ecological factors can have an overriding effect on genetic distance. Few studies have empirically demonstrated that IBE is influential in spatial patterns of genetic structure in *Plethodon* (e.g., Fisher-Reid et al. 2013). Kozak and Wiens (2006, 2010) hypothesized that population divergence and speciation in this genus has been decoupled from climatic niche divergence and that niche conservatism in this group is a major driver of species formation. However, our results agree with an analysis focused on the members of the *P. glutinosus* complex (Wooten et al. 2013), which found that different species in this particular group occupy different climatic niches.

The significant climate PCs (PC1 and PC4) were both strongly influenced by measures of temperature, although PC1 also included considerable influence from several precipitation variables. One of the most important variables was precipitation during the warmest quarter, indicating a synergistic effect of precipitation and temperature. The ability for salamanders of the genus *Plethodon*, which are lungless, to effect gas exchange is negatively impacted by warm temperatures; this is particularly the case for larger species such as members of the *P. glutinosus* complex (Feder 1983). Moreover, reproductive output of at least some members of this complex is influenced by precipitation (Milanovich et al. 2006). Our study region is characterized by fairly warm temperatures and multi-year droughts that occur every 25 years on average (Edwards et al. 2013). These combined effects may depress densities of individual populations, in turn exposing them to the homogenizing effects of genetic drift. If drift is a strong factor affecting these salamanders, the randomness of its influence among populations would be predicted to increase genetic divergence among them.

We also uncovered a significant association between NDVI and genetic divergence. NDVI measures differential light reflectivity caused by plant cover and, consequentially, measures greenness across the landscape. NDVI, therefore, can be used to measure the current state of landscape fragmentation, which significantly affects gene flow and subsequent patterns of genetic divergence (Noël et al. 2007; Dixo et al. 2009; Delaney et al. 2010). NDVI values are highly variable across our study area, with areas of native forest vegetation restricted to riparian corridors and small patches of intact habitat nested within a matrix dominated by deforested agricultural lands. The significant, negative relationship between NDVI and genetic divergence in our dataset suggests that salamanders occurring in habitats with more similar vegetative contexts also have higher amounts of genetic divergence. Given the positioning of our collection localities relative to the aforementioned state of habitat fragmentation across our study area, this relationship is likely the result of individual salamander populations persisting in these remaining areas of forest, which are found in small, relatively isolated patches separated by heavily disturbed areas within the larger landscape matrix. As such, this relationship is likely a signature of the influence of landscape fragmentation across the Coastal Plain.

While urbanization has been implicated as a primary cause of fragmentation and genetic isolation in the modern world (Noël et al. 2007; Delaney et al. 2010), fragmentation as a result of agricultural land practices, both past and present, is also influential (Vellend 2004). Dating to the late eighteenth and early nineteenth centuries, the lower Piedmont and Coastal Plain of the southeastern United States has a long history of extensive forest removal for timber and row-crop agriculture, especially cotton monoculture (Camp 1985). This loss of forest cover left a signature across the region that persists through a combination of continued row-crop farming, conversion of old fields to pastureland, and secondary succession slowed by a widespread loss of topsoil

(Wharton 1977). Indeed, work within our study system's broader physiographic context (Brudvig et al. 2013; Cox and Hart 2015) and elsewhere across the eastern United States (Orwig and Abrams 1994; Hall et al. 2002) has found that historical land use legacies, even those dating back multiple centuries, play a fundamental role in influencing variation in present-day land use and vegetation patterns across the landscape. NDVI is a measure of contemporary vegetation cover, although the latter itself is likely reflective of much older patterns of land use across our study region. Contemporary patterns in vegetation may also be driven by variation in slope and terrain, but the influence of those variables is difficult to distinguish in our study system since those same topographical factors also likely influenced the historic placement and development of agricultural lands and other human land use types in the Coastal Plain. We hypothesize that the significant relationship between NDVI and genetic distance in our analysis reflects the current and past land use history of the study region and that the patchiness of the landscape has contributed to genetic patterns in these salamanders. While we treated NDVI as a measure of environmental differences, the aforementioned patterns also suggest an influence of isolation by resistance (IBR), which has been demonstrated in this species complex (Peterman et al. 2014). Future work focusing more intensively on the influence of land use patterns and landscape fragmentation on genetic divergence among lungless salamanders in the Coastal Plain may further clarify this relationship.

The absence of a significant role of geographic distance, and the corresponding lack of support for the hypothesis of IBD, was surprising given the tendency of salamanders of this genus to be dispersal-limited (Marsh et al. 2004; Cabe et al. 2007; Ousterhout and Liebgold 2010). Moreover, previous studies have found IBD to be important to *Plethodon* divergence (Gibbs 1998; Cabe et al. 2007; Shepard and Burbrink 2011). Although sampling scale can

influence the strength of relationships in landscape genetics (Cushman and Landguth 2010), scale would not seem to be an issue here because IBD has been demonstrated in *Plethodon* at both fine (Gibbs 1998; Cabe et al. 2007) and coarse scales (Weisrock and Larson 2006; Shepard and Burbrink 2011). One thing that does affect the strength of IBD is the complexity of the landscape (Gibbs 1998) and the relative role of other factors, whose strength may override the effect of IBD in *Plethodon* (Weisrock and Larson 2006). This may be the case with *Plethodon* across the lower Piedmont and Coastal Plain of the southeastern United States; certainly our results suggest that the combined effects of IBE are more important.

Another unexpected outcome of our analysis was the absence of support for the hypothesis of IBB; there was no significant relationship between streams of 4th and higher orders and genetic divergence in Slimy Salamanders. *Plethodon* species virtually never enter water voluntarily (Jaeger 1986), and even small streams impede gene flow (Marsh et al. 2007). Moreover, several pairs of closely related species (*P. cinereus* and *P. serratus* Highton and Webster, 1976; *P. cylindraceus* and *P. teyahalee* Highton, 1989) are separated by the French Broad River. Therefore, sizeable streams would be expected to be related to genetic divergence in these salamanders. One hypothesis that would explain *Plethodon* gene flow across large streams of the Coastal Plain arises from the relatively soft, sedimentary nature of the substrate and the consequent tendency of these rivers to form and cut off meanders, forming oxbow lakes (Constantine and Dunne 2010). Large meanders, which may surround suitable *Plethodon* habitat in bottomland hardwoods (Petranka 1998) may be cut off, effectively moving the river to the other side of the salamanders. Such course changes are common in rivers of the southeastern Coastal Plain (Hupp 2000). Recent studies have also shown that the location and restructuring of ancient river courses explains current genetic patterns within certain lungless salamanders better

than the present-day location of rivers (Kozak et al. 2006a; Kuchta et al. 2016). Changes in stream courses may have worked in conjunction with IBE to have overridden any signature of IBB associated with streams.

Shifting stream courses would also explain the existence of the population referable morphologically and genetically to *P. chlorobryonis* on the western side of the Savannah River (eastern Burke County). This population grouped with *P. chlorobryonis/variolatus* in our haplotype networks, whereas those from the same side of the Savannah in western Burke County <25 km away did not. A similar situation has been reported along the Illinois-Missouri border, at which point the Mississippi River apparently shifted course and trapped a population of one parapatric species of Slimy Salamander (*P. glutinosus*) on the side otherwise occupied by a different species (*P. albagula* Shepard and Kuhns, 2018).

The relatively low proportion (approximately 15%) of genetic divergence explained by our MMRR model suggests that there are additional factors that influence divergence in Slimy Salamanders. Perhaps these include random effects of mutation and drift that are independent of landscape variables. What is clear from our analysis, however, is that the environment appears to contribute to genetic divergence among Slimy Salamanders in the lower Piedmont and Coastal Plain of the southeastern United States. Moreover, the considerable amount of genetic divergence among populations and the association with contemporary (or at least very recent) environmental conditions suggest that such environmental features continue to strongly influence genetic differentiation in these salamanders. Our results agree with earlier studies (Shepard and Burbrink 2008; Peterman et al. 2014) in that populations of these salamanders diverge in response to a complexity of landscape variables, not just to the traditionally viewed geographic ones (i.e., barriers and distance).

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Table 1. Localities for specimens of the *Plethodon glutinosus* complex collected for landscape analysis of genetic divergence (dashes indicate multiple specimens).

Specimen(s) ID	State	County	Latitude	Longitude
AP1—2	Georgia	Appling	31.92	-82.31
AT1—2	Georgia	Atkinson	31.20	-82.99
BL1—3	Georgia	Bleckley	32.45	-83.45
BR1—2	Georgia	Bryan	31.15	-81.78
BI1—2	Georgia	Bibb	32.85	-83.77
BKa1—3	Georgia	Burke	32.95	-82.07
BKb1—2	Georgia	Burke	33.09	-81.76
BKc1	Georgia	Burke	33.24	-81.94
BUa1—3	Georgia	Bulloch	32.43	-81.77
Bub1—2	Georgia	Bulloch	32.43	-81.78
BW1—2	Georgia	Baldwin	33.12	-83.18
BY1	Georgia	Brantley	31.11	-81.96
CA1—3	Georgia	Candler	32.33	-82.23
CB1	Georgia	Columbia	33.48	-82.28
CH1—2	Georgia	Chatham	32.05	-81.28
CL1	Georgia	Clarke	33.90	-83.36
CM1	Georgia	Camden	30.94	-81.67
CO1—2	Georgia	Coffee	31.91	-82.31
CP1	Georgia	Crisp	31.96	-83.91
DL1	Georgia	Dooley	32.13	-84.00

DO1	Georgia	Dodge	32.16	-83.14
DOb1	Georgia	Dodge	32.21	-83.16
ECa1	Georgia	Echols	30.72	-83.09
ECb1—2	Georgia	Echols	30.60	-82.71
EM1—2	Georgia	Emanuel	32.61	-82.43
EFa1—3	Georgia	Effingham	32.34	-81.32
EVb1—2	Georgia	Effingham	32.34	-81.47
EFc1	Georgia	Effingham	32.58	-81.38
GR1	Georgia	Greene	33.72	-83.29
HO1	Georgia	Houston	32.49	-83.60
HP1—2	South Carolina	Hampton	32.59	-81.25
HPa1—3	South Carolina	Hampton	32.80	-80.90
HSa1	Georgia	Harris	32.77	-85.08
HSb1	Georgia	Harris	32.83	-84.81
IR1—2	Georgia	Irwin	31.50	-83.38
JE1—2	Georgia	Jefferson	32.87	-82.42
JS1	Georgia	Jasper	33.30	-83.66
LA1	Georgia	Laurens	32.41	-82.74
LA2	Georgia	Laurens	32.68	-82.97
LG1—2	Georgia	Liberty	31.91	-81.56
LR1—3	Georgia	Lanier	30.93	-83.02
MA1	Georgia	Madison	34.18	-83.13
MC1	Georgia	McDuffie	33.38	-82.42

ME1	Georgia	Meriwether	33.10	-84.53
MG1—2	Georgia	Montgomery	32.32	-82.43
MH1—2	Georgia	McIntosh	32.55	-81.40
MN1	Georgia	Monroe	33.03	-83.81
NA1—2	Florida	Nassau	30.78	-81.96
PC1	Georgia	Pierce	31.29	-82.37
PK1	Georgia	Pulaski	32.26	-83.40
PU1	Georgia	Putnam	33.34	-83.16
SC1—2	Georgia	Screven	32.85	-81.51
SU1—2	Georgia	Sumter	32.05	-84.22
TA1—2	Georgia	Tattnall	31.87	-82.05
TB1	Georgia	Talbot	32.54	-84.58
TE1	Georgia	Telfair	31.84	-82.81
TR1—2	Georgia	Treutlen	32.36	-82.72
TY1	Georgia	Taylor	32.57	-84.28
UP1	Georgia	Upson	32.86	-84.49
UP2	Georgia	Upson	32.88	-84.47
WA1—3	Georgia	Wayne	31.48	-81.64
WE1—3	Georgia	Ware	31.13	-82.51
WH1	Georgia	Wheeler	32.25	-82.65
WH-TYPE1—2	Georgia	Wheeler	32.10	-82.90
WK1	Georgia	Wilkinson	32.81	-83.33
WS1	Georgia	Washington	33.02	-82.98

WX1—2	Georgia	Wilcox	32.85	-83.21
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Table 2. Localities for additional specimens of the Slimy Salamander (*Plethodon glutinosus*) complex collected for genetic context (dashes indicate multiple specimens). Species ID based on geographic ranges delimited by Highton (1989).

Specimen(s) ID	State	County	Species ID	Latitude	Longitude
BB1—2	South Carolina	Berkeley	<i>variolatus</i>	33.18	-79.50
BCNC	North Carolina	Bladen	<i>chlorobryonis</i>	34.53	-78.28
FU1	Georgia	Fulton	<i>glutinosus</i>	33.57	-84.71
HA1	Georgia	Habersham	<i>chlorobryonis</i>	34.56	-83.54
SAFL1—2	Florida	Santa Rosa	<i>grobmani</i>	30.71	-87.03
WLb1—2	Georgia	Walker	<i>glutinosus</i>	34.71	-85.36

Draft

Table 3. Surrogate variables used to test hypotheses of landscape effects on genetic divergence of Slimy Salamanders (*Plethodon glutinosus* species complex) in the lower Piedmont and Coastal Plain of the southeastern United States. IBD = isolation by distance; IBE = isolation by environment; IBB = isolation by barrier.

Hypothesis	Measured variable
IBD	Geographic distance
IBB	4 th -order streams
IBE	Principal components of 19 climatic variables from BioClim Normalized Difference Vegetation Index (NDVI) Watershed

Table 4. WorldClim variables (<http://www.worldclim.org>) used for testing the influence of isolation by environment on members of the Slimy Salamander (*Plethodon glutinosus*) complex across the lower Piedmont and Coastal Plain of the southeastern United States.

Variable	Description
BIO1	Mean annual temperature
BIO2	Mean diurnal temperature range
BIO3	Isothermality
BIO4	Seasonality of temperature
BIO5	Maximum temperature during warmest period
BIO6	Minimum temperature during coldest period
BIO7	Annual temperature range
BIO8	Mean temperature during wettest quarter
BIO9	Mean temperature during driest quarter
BIO10	Mean temperature during warmest quarter
BIO11	Mean temperature during coldest quarter
BIO12	Annual precipitation
BIO13	Precipitation during wettest period
BIO14	Precipitation during driest period
BIO15	Standard deviation of means of weekly precipitation
BIO16	Precipitation during wettest quarter
BIO17	Precipitation during driest quarter
BIO18	Precipitation during warmest quarter
BIO19	Precipitation during driest quarter

Table 5. Factor scores for principal components (PCs) from principal components analysis (PCA) of 19 climate variables (percentage of variance explained by each PC in parentheses).

Variable	PC1 (58.9)	PC2 (20.1)	PC3 (8.3)	PC4 (6.3)
BIO1	0.951	-0.260	0.048	0.118
BIO2	-0.569	-0.492	0.621	-0.169
BIO3	0.301	-0.447	0.819	0.084
BIO4	-0.960	-0.008	-0.212	-0.109
BIO5	0.343	-0.801	0.201	-0.261
BIO6	0.973	-0.080	-0.017	0.158
BIO7	-0.920	-0.210	0.093	-0.267
BIO8	0.741	-0.258	0.013	-0.409
BIO9	-0.519	-0.358	0.064	0.623
BIO10	0.815	-0.503	-0.134	0.068
BIO11	0.970	-0.159	0.097	0.115
BIO12	0.419	0.801	0.336	0.198
BIO13	0.779	0.578	0.167	0.020
BIO14	-0.560	0.576	0.276	-0.345
BIO15	0.965	0.185	-0.022	0.017
BIO16	0.813	0.539	0.149	-0.031
BIO17	-0.609	0.685	0.289	-0.075
BIO18	0.951	0.216	0.103	-0.149
BIO19	-0.812	0.059	0.255	0.462

Table 6. Results of landscape variable analysis using Multiple Matrix Regression with Randomization (F for model = 120.9376; P = 0.001).

Variable	Coefficient	<i>t</i> statistic	<i>P</i>
Model intercept	0.3122	21.5675	0.015
Species	-0.1899	-19.1618	0.001
Climate PC1	0.1165	5.256	0.007
Climate PC4	0.3568	17.9960	0.001
NDVI	-0.01483	-8.2272	0.009
Geographic distance	-0.0242	-08299	0.720
Watersheds	0.0101	0.6603	0.614
Streams	0.0189	1.2150	0.505
Climate PC2	0.0385	2.2866	0.383
Climate PC3	-0.0814	-5.0255	0.061

Fig. 1. Localities for members of the *Plethodon glutinosus* complex. Black dots represent localities for collections made for this study. Black dots enclosed within the circle represent localities from which specimens were collected that were used for analysis of landscape genetics. Black dots outside the circle represent localities from which specimens were collected as representatives of named taxa. Other symbols represent localities for named taxa reported by Highton (1989).

Fig. 2. Network of haplotypes for three segments of the mitochondrial genome used in genetic analysis of the *Plethodon glutinosus* complex generated in Network version 5.0.

Fig. 3. Network of haplotypes for the nuclear intron *ILF3* used in genetic analysis of the *Plethodon glutinosus* complex generated in Network version 5.0.