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Variation in Head Shape and Color at the Range Boundary of Gulf Coastal Slimy Salamanders (*Plethodon glutinosus* Complex), USA

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Taxonomists currently recognize 16 cryptic species within the *Plethodon glutinosus* complex revealed by allozyme analysis but that typically do not differ in morphology or color pattern. Two putative species, *P. grobmani* and *P. mississippi*, are distributed across the Gulf Coastal Plain, with geographic ranges that are separated by Mobile Bay and the Alabama River. Character divergence is thought to distinguish the two species, with *P. grobmani* (east of Mobile Bay) having a short snout and extensive patches of white coloration, and *P. mississippi* (west of Mobile Bay) having an elongate snout and lacking extensive patches of white coloration. However, specimens used to characterize these two species were examined in life, were collected mainly from areas adjacent to Mobile Bay, and were evaluated by a single investigator. Additionally, the two traits (snout shape and color) were combined into a single variable, masking the contribution of each trait to individuating the two species. To test the utility of these characters, we applied identical measurements of the two traits to preserved specimens representing an east–west transect across the geographic ranges of the two putative species. Data were generated by three investigators who measured each specimen twice, a design that allowed examination of reproducibility within and among investigators. Traits were evaluated separately and as a combined score. Both snout shape and color were found to be traits that are reproducible for measurements within individual investigators and the measurement of these traits did not differ among investigators. Specimens from east of Mobile Bay had more extensive white coloration and higher total scores than those west of Mobile Bay; snout shape did not differ across Mobile Bay. Longitude was a significant correlate of color and total score, with the slope of the relationship differing on each side of Mobile Bay. Color and total score of specimens west of Mobile Bay had a positive association with longitude, while scores of specimens east of Mobile Bay had a negative association. Thus, rather than exhibiting character divergence across Mobile Bay, slimy salamanders converge on a phenotype with extensive white coloration at Mobile Bay. We find no color or morphological feature that distinguishes putative *P. grobmani* from putative *P. mississippi*, a line of evidence suggesting that those populations are a single species with regional change in color.

EXAMINATION of changes in character states across species boundaries continues to provide vital evidence for delimiting species and examining processes of speciation. Some isolated populations appear to represent single species, despite great geographic isolation, because key variables associated with reproductive isolation show limited evidence of differentiation (e.g., Warwick et al., 2015). In other cases, adjacent populations appear to represent multiple species, despite limited levels of genetic differentiation, because key variables associated with reproductive isolation differ (e.g., Peakall and Whitehead, 2014). In the latter cases, character displacement may operate to limit gene flow between species while maintaining it within species (e.g., Lemmon, 2009). Additionally, when characteristics of populations change abruptly at a geographic boundary, even when the characters are of unknown relevance to gene flow, sufficient evidence for species recognition can be reached (e.g., Ennen et al., 2010).

Many of the most challenging problems in taxonomic herpetology involve populations for which molecular evidence of lineage formation lacks additional supportive evidence of divergence. For example, taxonomy of slimy salamanders (*Plethodon glutinosus* complex) has been guided by species delimited within Highton (1989). That pioneering work used phenetic analysis of allozymes to reveal 16 species from what had been viewed as a single, wide-ranging species, with 14 of these constituting the *P. glutinosus* complex (Highton et al., 2012) and the other two representing species described earlier (*P. aureolus*; Highton, 1984) or resurrected (*P. kentucki*; Highton and McGregor, 1983). As a result, the complex has been widely espoused to be an example of cryptic diversity revealed through molecular analyses of a morphologically conservative group (e.g., Highton, 1995; Wiens et al., 2006). Highton (1989) noted that identification of these species was hampered because most lacked autapomorphic features, either for traditional morphological and color features or among allozymes. However, the demonstration that two species, *P. glutinosus* and *P. teyahalee*, lack gene flow where they overlap in distribution (Highton, 1990) suggested that eventually evidence would reveal the other lineages of Highton (1989) to be distinct.

Additional evidence of divergence within the *P. glutinosus* complex has not emerged from examination of morphology across the complex. Carr (1996) examined morphological variation from 26 populations representing 13 species of the *P. glutinosus* complex plus *P. kentucki*. Discriminant function analyses found that most variation (75%) among populations was explained by body size, which separated small-bodied populations of the Coastal Plain from upland populations of larger body size. These discriminant functions performed poorly at classifying species erected by Highton (1989), and Carr (1996) hypothesized that speciation occurred so recently that accompanying morphological divergence had not yet occurred. Similarly, phylogenetic relationships of these species estimated from mitochondrial and nuclear genes recovered trees with short branch lengths, weak node support, and rampant evidence of introgression (Wiens et al., 2006; Highton et al., 2012; Fisher-Reid et al., 2013). These results have been interpreted to indicate niche conservatism within a non-adaptive radiation, in which lineage origination occurs at a more rapid rate than evolution of reproductive isolation (Kozak et al., 2006; Wiens et al.,

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2006). However, because the species of the *P. glutinosus* complex are morphologically and ecologically indistinguishable, it becomes difficult to determine whether several species have individuated or whether metapopulation structure within a single species has been revealed by the analyses (Wiens et al., 2006). In such a situation, development of complementary evidence differentiating adjacent populations, especially at their geographic boundary, becomes vital (Dayrat, 2005; Will et al., 2005).

One species pair within the *P. glutinosus* complex appears to show color and morphological differences across a geographic barrier separating the pair. Lazell (1994) examined the Florida slimy (*Plethodon grobmani*) and Mississippi slimy (*Plethodon mississippi*) at their contact zone across Mobile Bay in southern Alabama and Mississippi (Highton, 1989). Samples of these taxa were used to test for diagnostic morphological and color differences for each species. Based on a total score of morphology and color, a near-perfect separation of the putative species was documented. These results suggested that *P. mississippi* is characterized by an elongate snout and dark color pattern and *P. grobmani* by a short snout and white color categories for all body regions examined, either through character divergence at the geographic barrier separating the species pair or uniform differences between the species across their geographic ranges.

The results of Lazell (1994) provided some hope that *P. grobmani* and *P. mississippi* can be reliably separated from each other. However, it is unclear whether morphology or color alone separate the two taxa or whether both characters must be used. Additionally, the specimens sampled were relatively limited in geographic distribution: two sites in Baldwin County, Alabama, and one site in Mobile County, Alabama (Mon Louis Island), were assumed to be *P. grobmani*, while one site in Mobile County, Alabama, and two sites in Mississippi were assumed to be *P. mississippi*. Thus, the samples were largely from areas adjacent to the proposed dispersal barrier, making it unclear whether the differences attributed to the two species characterized the entire range of each species or represented character divergence at the geographic boundary. Lazell (1994) also failed to determine how reproducible the color and morphology data were, an important issue given that specimens from Mon Louis Island, a continental island in Mobile County, were assumed to be *P. grobmani* based on visual inspection, despite being on the wrong side of the putative barrier between the two species. Finally, the characters were not tested on preserved specimens as a possible mechanism for identifying museum specimens. This would be of particular interest if color and morphology were to be used to delimit allozyme species in a more integrative taxonomic framework (i.e., Dayrat, 2005; Will et al., 2005).

To this end, we used museum specimens of *P. grobmani* and *P. mississippi* along a longitudinal transect across Mobile Bay, Alabama, to explore patterns of snout shape and color between the species. From these specimens, we sought to determine whether color and snout shape measurements were reproducible within and among investigators, and if those measures supported character differences across Mobile Bay, the proposed barrier separating the two putative taxa.

MATERIALS AND METHODS

We examined specimens of the *P. glutinosus* complex housed in the research collections of the Auburn University Museum of Natural History. The specimens were selected because they form an east–west transect from Alachua County, Florida, to Marion County, Mississippi, with 51 specimens occurring east of Mobile Bay and 11 specimens occurring west of the bay (Fig. 1). For each specimen, we recorded the latitude and longitude of its collection locality and took three images of the body, illuminated from a copy stand. One image was of the top of the head and neck taken perpendicular to the specimen, one was of the chin and arms, and one was of the left side of the body. Each image included a ruler for scale. The image of the top of the head was used to measure snout width (distance between medial corner of right and left eyes) and the distance from the tip of the snout to a line connecting the medial corner of each eye (as in figure 2 of Lazell, 1994). Photoshop 6.0 or ImageJ were used for both measurements and to generate the interorbital line.

Color variables were coded from the ventral and lateral images, following the protocol from Lazell (1994). For the sides of the body, color was categorized on a scale from 0 (less than 50% of lateral surface white) to 3 (lateral surface white except for reticulum of black; Fig. 2A). For the chin, categories ranged from 0 (no white on gular fold) to 3 (wide white border along gular fold; Fig. 2B). Separate scores were recorded for the left and right axilla, with categories ranging from 0 (no white border) to 4 (wide white border; Fig. 2C).

Snout shape was used to characterize morphology. For this variable, snout width was divided by snout length. This ratio was then multiplied by 10. A total score was generated by summing the score for snout shape and the four color scores, as in Lazell (1994). When the components of the total score were generated, only the specimen number identified each image. Thus, observers were blind to the actual identity or locality of each specimen, eliminating any consistent bias in judging scores. All measures were taken by three individuals (BF, SMG, and CG) to assess inter-investigator variation, and these measurements were retaken for each specimen 3–14 days later to assess reproducibility.

We first used a one-way ANCOVA to test variation among investigators. For each investigator, the second observation of a specimen was regressed on the first observation of that specimen. This analysis allowed us to examine inter-investigator bias and reproducibility. F-tests for both slope and elevation were used to examine inter-investigator variation of total, snout, and color scores. Because we expected readings to be reproducible, we tested our regressions against an expected line with slope of 1.0 (regressions forced through the origin). Observations from all three investigators were pooled and used to estimate slopes of regressions for total, snout, and color scores. Confidence limits (95%) of slopes were used to assess whether observed values differed significantly from the expected value. Snout scores were ratio-scale data that conformed to the bivariate normal distribution required for parametric methods. Color scores were ranked categories that, when summed across all regions, ranged from 0–14. Residuals of regressions involving these scores were examined via q-q plots. Because these residuals displayed a tight fit to the expected values of bivariate normal variables, we analyzed these data with parametric tests and did the same for color scores added to snout scores.

Once inter-investigator variation was evaluated, we used one-way ANCOVA to examine the effect of location (east vs. west of Mobile Bay) on specimen measurements. Here, mean values of all six observations of each specimen (3 observers x 2 replicate observations) were used as response variables.

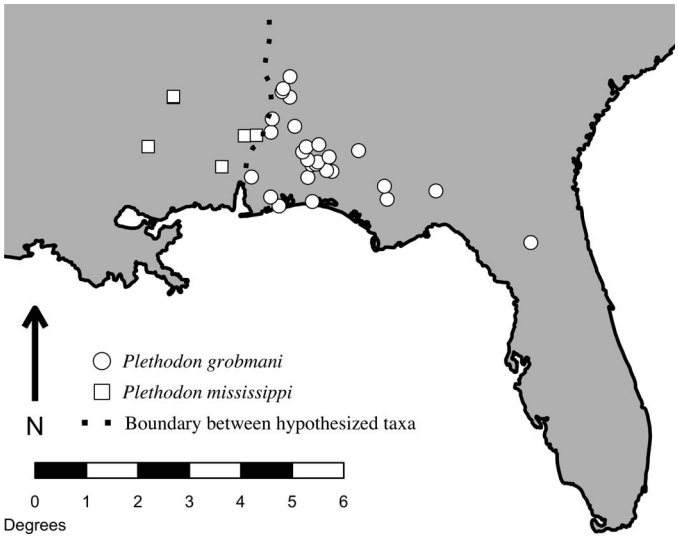

Fig. 1. Map of localities in the southeastern United States for museum specimens of slimy salamanders (*Plethodon*) examined in the study. The dashed line indicates the hypothetical barrier between the taxa *Plethodon grobmani* and *Plethodon mississippi* in Alabama.

Longitude was included in the model as a covariate to ensure that geographic differences were not evident, other than the location main effect. Because Lazell (1994) indicated that scores for specimens east of Mobile Bay should have higher scores than those west of the bay, a one-tailed statistical hypothesis was assessed. *F*-tests of each variable (total, snout, and color scores) were used to determine whether mean values differed significantly between locations. When longitude was revealed to be a significant correlate of the three variables and a significant interaction of longitude and location characterized these data, least-squares linear regressions were used to summarize patterns east and west of Mobile Bay. All statistical analyses were performed in SAS 9.4 (SAS Institute, Cary, NC) with $\alpha = 0.05$. For tests of differences between the two locations, the same overall hypothesis was examined for combined scores, color scores, and snout scores. In these tests, *P*-values were adjusted to control for family-wise error rates by the bootstrap-resampling method of Yekateuli and Benjamini (1999).

RESULTS

Regressions of the second total score on the first did not differ significantly among investigators ($F_{2,180} = 0.43$, $P = 0.65$). When pooled across all three investigators, regression of the second total score on the first had a significant slope ($F_{1,180} = 36870.50$, $P < 0.0001$; Fig. 3A) that did not differ from 1.0 (95% CL = 0.981–1.001). Similarly, regressions of the second snout score on the first and the second color score on the first did not differ significantly among investigators ($F_{2,180} = 2.89$, $P = 0.06$ for snout score; $F_{2,180} = 0.29$, $P = 0.75$ for color score). When data were pooled for all investigators, regression of the second observation on the first yielded a relationship with a significant slope for snout ($F_{1,180} = 49434.60$, $P < 0.0001$; Fig. 3B) and color score ($F_{1,180} = 5636.34$, $P < 0.0001$; Fig. 3C); the slope did not differ from 1.0 for snout score (95% CL = 0.996–1.014) and approached 1.0 for color score (95% CL = 0.924–0.974).

Total scores were significantly higher for specimens east of Mobile Bay relative to those west of the bay ($F_{1,61} = 14.39$, $P = 0.0006$). However, the effect of longitude on total score differed significantly between locations ($F_{1,61} = 14.70$, $P = 0.0008$) with longitude having a significant negative association with total score east of Mobile Bay (y = –0.98x–55.78, $R^2 = 0.17$, $F_{1,49} = 9.82$, $P = 0.002$) and a significant positive association west of the bay (y = 3.05x+297.00, $R^2 = 0.42$, $F_{1,9} = 6.47$, $P = 0.02$; Fig. 4A). Snout scores were not significantly higher for specimens east of Mobile Bay relative to those west of the bay ($F_{1,61} = 2.32$, $P = 0.13$), and there was no interaction of longitude and snout score ($F_{1,61} = 2.36$, $P = 0.13$) because longitude was not a significant predictor of this variable either east of Mobile Bay (y = –0.21x–21.09, $R^2 = 0.02$, $F_{1,49} = 0.75$, $P = 0.20$) or west of it (y = 1.01x+109.86, $R^2 = 0.15$, $F_{1,9} = 1.58$, $P = 0.12$; Fig. 4B). Color scores were significantly higher for specimens east of Mobile Bay compared with those west of the bay ($F_{1,61} = 11.96$, $P = 0.0008$). However, the effect of longitude on color score differed significantly between locations ($F_{1,61} = 12.23$, $P = 0.0008$), with longitude having a significant negative association with color score east of Mobile Bay (y = –0.80x–60.75, $R^2 = 0.17$, $F_{1,49} = 9.91$, $P = 0.0014$) and a significant positive association west of the bay (y = 2.03x+187.15, $R^2 = 0.46$, $F_{1,9} = 7.55$, $P = 0.01$; Fig. 4C).

DISCUSSION

If morphological data summarized by Lazell (1994) reinforced allozyme data in revealing speciation across Mobile Bay, then we expected either to recover character divergence for specimens on either side of this barrier or uniform differences across the entire range of each species. Instead, our data reveal character convergence across Mobile Bay. Specimens of putative *P. grobmani* from as far east as Alachua County, Florida, have significantly lower total and color scores as do specimens of putative *P. mississippi* from as far west as Marion County, Mississippi. These scores increase as putative *P. grobmani* approach Mobile Bay from the east and putative *P. mississippi* approach the bay from the west, creating a phenotype with extensive white coloration across a broad swath between the western side of Mobile Bay (ca. –89.00 longitude) and the Apalachicola River (ca. –86.00 longitude). The area of the Coastal Plain between the Mobile and Apalachicola rivers in eastern North America is a globally significant biodiversity hotspot (Noss et al., 2015) that harbors a growing number of cryptic species (e.g., *Sternotherus intermedius*; Scott et al., 2018), mitochondrial lineages (e.g., *Scincella lateralis* Panhandle clade; Jackson and Austin, 2012), and subspecies (e.g., *Graptemys nigrinoda delticola*; Guyer et al., 2015). We reject the hypothesis that this colorful phenotype represents a distinct species because evidence of monophyly is lacking (Joyce et al., 2019, in this volume). Instead, selection or neutral processes in this region appear to have derived the relatively bold color pattern observed here relative to populations at either longitudinal extreme. Importantly, the presence of this phenotype on both sides of Mobile Bay and a lack of monophyly for specimens attributed to *P. grobmani* and *P. mississippi* (Joyce et al., 2019, in this volume) reject Mobile Bay as a dispersal barrier separating the two species assumed by Lazell (1994).

Like Lazell (1994), we found significantly higher total scores for specimens east of Mobile Bay compared to those west of the bay. However, while Lazell (1994) reported virtually no overlap of total score (18–30 for putative *P. mississippi*; 30–43 for putative *P. grobmani*) between populations across the bay, we found extensive overlap between the two locations (22–35 for *P. mississippi*; 22–34 for *P. grobmani*).

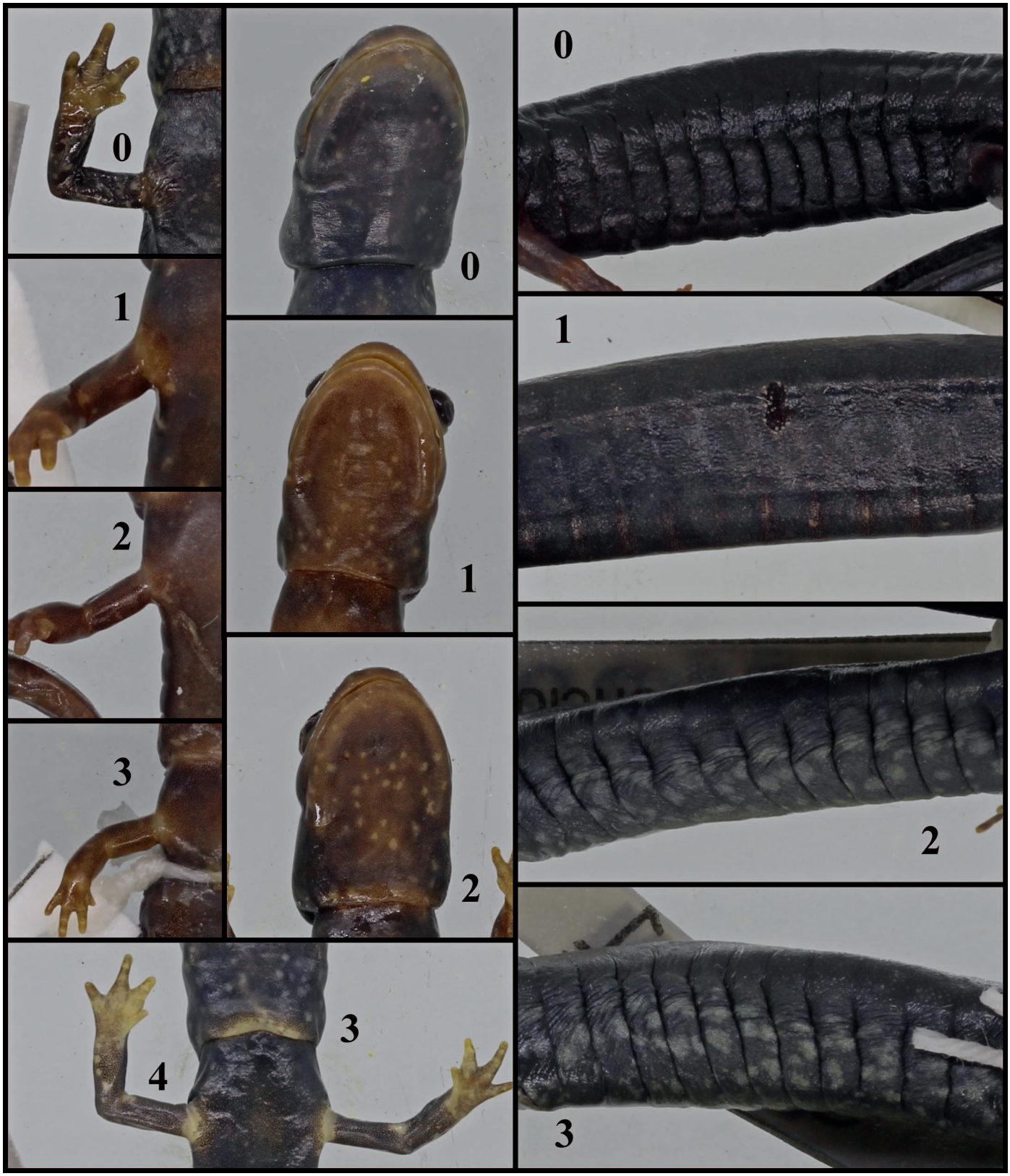

Fig. 2. Ranked categories used to evaluate variation in white coloration of slimy salamanders (*Plethodon*) examined in the study: the left axilla (left column; 0–4), gular fold (central column; 0–3), and lateral aspect of the body column (right column; 0–3). Ranks ranged from no or limited white coloration (0–1) to extensive white coloration (3 or 4), following Lazell (1994).

We argue that this difference emerges from Lazell's (1994) decision to include specimens from Mon Louis Island in his concept of *P. grobmani*. Mon Louis is in the southeast corner of Mobile County on the west side of Mobile Bay. Lazell (1994) considered specimens from this site to be *P. grobmani* because they had extensive white coloration and were described to have short snouts, like those of specimens from Baldwin County on the east side of Mobile Bay. However,

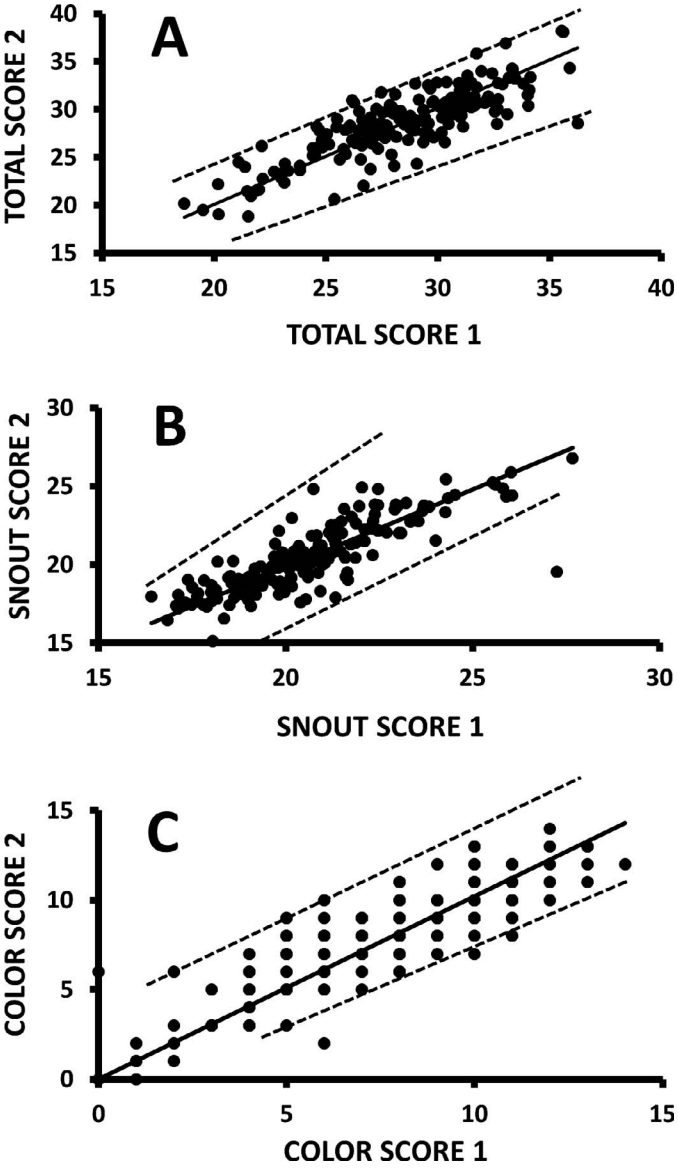

Fig. 3. Regression of second observation on first observation for total (A), snout (B), and color (C) scores. Solid line is ordinary least-squares linear regression; dashed lines indicate 95% prediction limits. See text for statistics.

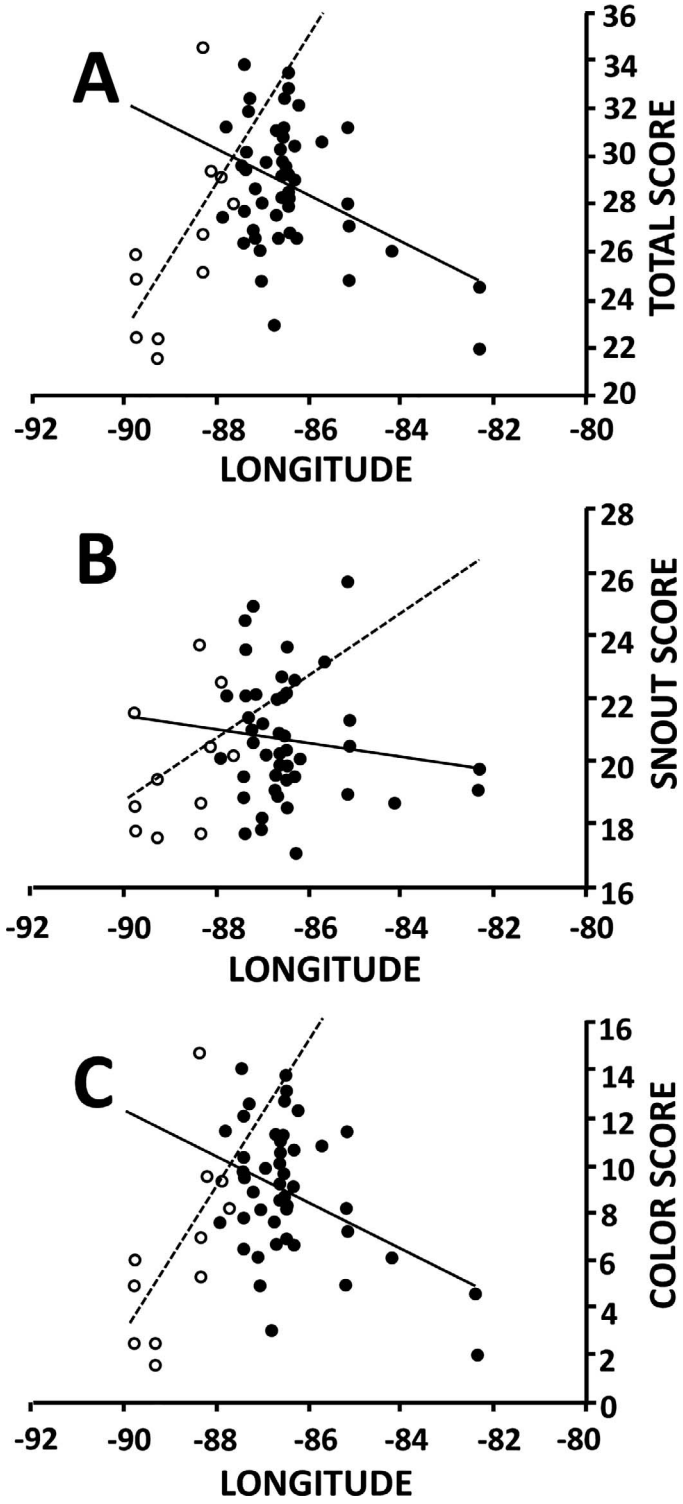

Fig. 4. Regression of total (A), snout (B), and color (C) scores on longitude for slimy salamanders east (solid circles; solid line) and west (open circles; dashed line) of Mobile Bay in Alabama. See text for statistics.

because none of the specimens used in that study was examined via electrophoresis, there was no way to confirm the electrophoretic identity of the species generated by Highton (1989). This problem was exacerbated because 13 of the 25 specimens that Lazell (1994) considered to be *P. grobmani* were from a single vacant lot that comprised the Mon Louis specimens, a sampling regime that hampered his ability to characterize color and shape of this taxon. His concept of *P. mississippi* was based on specimens from one site in central Mobile County and two sites in eastern Mississippi, all of which lacked extensive white coloration and were described to have elongate snouts. However, our data, which include a much broader representation of the geographic ranges of the two taxa, document a statistically significant trend for putative *P. mississippi* to possess more extensive white coloration and a statistically insignificant trend for this putative species to possess shorter snouts along the western edge of Mobile Bay. Thus, specimens possessing the features used by Lazell (1994) to diagnose *P. grobmani* are not restricted to Mon Louis on the west side of Mobile Bay, but are found as far northward as Clarke County, Alabama, near a site sampled by Highton (1989) and used in the delimitation process defining the range of *P. mississippi*. We conclude that, had Lazell (1994) identified the Mon Louis specimens as *P. mississippi*, as was done in subsequent studies (e.g., Cunningham et al., 2009), the total score generated for

color and snout shape would have failed to reveal strong character separation for the two putative species.

Patterns of variation associated with total score reveal a broader range of values recovered by Lazell (1994; range of 18–43) than by us (range of 19–36), especially for the most colorful, short-snouted specimens. We suggest that this stems from differences in measures of snout shape rather than differences in color. Our evaluation of color recovers specimens with scores as low as 0 (no white color in any of the anatomical regions) to 14 (maximum degree of white color in all anatomical regions). Because we used categories defined in Lazell (1994), we infer that he recovered an identical range of color values from live specimens. This suggests that color patterns coded for specimens in preservative are similar to those coded in life. In fact, we found coding of the chin and axilla regions to be straightforward for preserved specimens because these regions retain colors as distinct as those shown in figure 3 of Lazell (1994). Lateral colors were more difficult to code because of fading that occurs in preservation; nevertheless, careful examination of the ventral half of this region revealed remnants of white color that are distinct enough to code into the categories described by Lazell (1994). Additionally, our analyses demonstrated that color variables from preserved specimens are reproducible and did not differ significantly among investigators. We take these results as indication that fading did not alter our ability to characterize color patterns in a fashion comparable to that of Lazell (1994).

Our results indicated that measures of snout shape were more difficult to code than those for color, as shown by differences among investigators that approached statistical significance. Nevertheless, those differences were reproducible, as indicated by a significant linear relationship between the first and second measures of this variable for all observers. Our snout scores ranged from 16 to 21, a narrower range than for color. Thus, our total score revealed more about variation in color than snout shape. Lee (1982) documented that preservation of amphibians alters morphological patterns observed in live specimens. We acknowledge that this factor likely explains differences in the range of total score observed between our samples and those of Lazell (1994). However, Lee (1982) also noted that relationships of linear variables in preserved versus fresh specimens tended to differ in elevation and not slope. This suggests that differences in snout shape between putative species remain in both living and preserved specimens but may become more difficult to detect in preservation.

Our failure to confirm color and morphological features distinguishing *P. grobmani* from *P. mississippi* causes us to question the utility and appropriateness of recognizing them as separate species. The proposed distribution maps for these two taxa have changed noticeably from the boundary-less maps of Highton (1989) to those of recent field guides (e.g., Powell et al., 2016), suggesting that the hypothetical distributions of the taxa are shifting unpredictably, despite no new data delimiting the species. Indeed, replicate samples of *P. grobmani* and *P. mississippi* failed to recover two monophyletic lineages when assessed with accumulating sequence data (Joyce et al., 2019, in this volume) and failed to conform to the geographic hypotheses for their distributions summarized in maps. Thus, we view the accumulating morphological and molecular sequence data as being most consistent with a single species of slimy salamander across the southern Coastal Plain, but with some process yielding colorful specimens from the Apalachicola to the Mobile drainages and with evidence of gene flow across both potential barriers.

MATERIAL EXAMINED

All specimen numbers listed below are from the herpetology collection at the Auburn University Museum of Natural History (AUM).

East of Mobile Bay: Alabama: 2772, 2779, 2803, 2804, 6974, 7550, 7551, 7552, 10057, 16783, 18586, 18587, 18588, 18643, 21888, 22349, 26766, 30074, 30880, 30881, 32104, 32105, 33764, 33765, 33766, 33767, 33768, 37781, 40819, 40820, 41513, 41552, 41851, 41852; Florida: 558, 640, 650, 2141, 2143, 2144, 2813, 2814, 13771, 27313, 27315, 27316, 30385, 30386, 30435, 35320, 40221.

West of Mobile Bay: Alabama: 12731, 15487, 15837, 15838, 15839; Mississippi: 2790, 20376, 20377, 34279, 39841, 39842.

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