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Clinal variation or validation of a subspecies? A case study of the *Graptemys nigrinoda* complex (Testudines: Emydidae)

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Widely distributed species often display intraspecific morphological variation due to the abiotic and biotic gradients experienced across their ranges. Historically, in many vertebrate taxa, such as birds and reptiles, these morphological differences within a species were used to delimit subspecies. *Graptemys nigrinoda* is an aquatic turtle species endemic to the Mobile Bay Basin. Colour pattern and morphological variability were used to describe a subspecies (*G. n. delticola*) from the lower reaches of the system, although it and the nominate subspecies also reportedly intergrade over a large portion of the range. Other researchers have suggested that these morphological differences merely reflect clinal variation. Our molecular data (mtDNA) did not support the existence of the subspecies, as the haplotypes were differentiated by only a few base pairs and one haplotype was shared between the putative subspecies. While there were significant morphological and pattern differences among putative specimens of *G. n. nigrinoda*, *G. n. delticola* and *G. n. nigrinoda* × *delticola*, these differences probably represent clinal variation as they were also related to environmental variables [i.e. cumulative drainage area and drainage (categorical)]. Specimens occupying slow-current, high-turbidity river reaches (e.g. the Tensaw River) exhibited greater relative carapace heights and more dark pigmentation, while specimens occupying fast-current, clearer rivers (e.g. the upper Alabama, Cahaba and Tallapoosa rivers) exhibited lower carapace heights and more yellow pigmentation. Given the absence of clear molecular and morphological differences that are related to drainage characteristics, we suggest that there is not sufficient evidence for the recognition of *G. n. delticola* as a distinct subspecies. *Biological Journal of the Linnean Society*, 2014, ••, ••–••.

ADDITIONAL KEYWORDS: intradrainage variability – morphometrics – plasticity.

INTRODUCTION

Geographical patterns of variation have long been the subject of study in evolutionary biology. How this variation originates and is maintained in continuously distributed populations has been of particular interest (Endler, 1977). Species distributed over large regions commonly experience a heterogeneous environment in terms of both abiotic and biotic components that vary not only spatially but also temporally. The interaction between natural selection and gene flow along these gradients may explain the maintenance of clinal variation in a trait with adaptive significance (Felsenstein, 1976). However, many

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processes may ultimately influence these patterns such as genetic drift, the amount of gene flow, the strength of the environmental gradient and interactions among loci (Endler, 1977).

Geographical variation within a species, especially in vertebrates, has often received taxonomic recognition through the designation of subspecies. In its many forms, the term 'subspecies' has been applied to capture incipient species, local adaptations and geographical variation within a species (reviewed by Manier, 2004), usually based upon morphological traits and colour variation (Haig *et al*., 2006). In many cases, these morphological characters are not phylogenetically relevant or they are incongruent with phylogenetic relationships derived from molecular data (Zink, 1989, 2004; Burbrink, Lawson & Slowinski, 2000). For example, subspecies are often mistakenly described based on morphological extremes along environmental gradients (Mulcahy, 2008), where delineations of the subspecies boundaries are arbitrary at best and do not reflect phylogenetic relationships. The numerous studies that document the presence of clinal variation in morphology along environmental gradients suggests that the taxonomy of many groups may be heavily impacted by this phenomenon (gastropods, Haase, 2003; Minton, Norwood & Hayes, 2008; bivalves, Watters, 1994; plants, Prentice, 1986; fishes, Langerhans & Reznick, 2010; Schaefer, Duvernell & Kreiser, 2011; mammals, Storz *et al*., 2001; Cardini, Jansson & Elton, 2007; birds, James, 1982; reptiles, Manier, 2004; amphibians, Gouveia *et al*., 2013). This is an important issue to resolve as a solid evolutionary basis for taxonomic designations is important in conservation planning given the scarcity of resources to manage threatened and endangered species (Haig *et al*., 2006; Moritz & Potter, 2013).

Graptemys is one of the largest and most taxonomically controversial North American turtle genera. Historically, the systematic relationships of species in the genus *Graptemys* have been supported largely by soft tissue and shell pigmentation patterns (Lovich & McCoy, 1992; Ernst & Lovich, 2009; Ennen *et al*., 2010a, b). Unfortunately, compared with other emydid genera, the genus *Graptemys* possesses shallow lineages and poor species-level resolution based on mitochondrial (mtDNA) and nuclear DNA (Lamb *et al*., 1994; Ennen *et al*., 2010b; Wiens, Kuczynski & Stephens, 2010). The disparity between the levels of variation in morphological traits and molecular data, in some cases, has perpetuated taxonomic uncertainties within the group (Walker & Avise, 1998). Contributing to the uncertainties are species whose descriptions are based on a limited number of characters that sometimes overlap and have not been statistically tested for significant morphological differentiation (e.g. *Graptemys flavimaculata*; Ennen *et al*., 2010a). This same problem is manifested in the description of a subspecies of *Graptemys nigrinoda* in the Mobile Bay Basin (Folkerts & Mount, 1969).

In the original species description of *Graptemys nigrinoda*, Cagle (1954) recognized morphological variation within the species between the upper and lower Alabama populations. However, these characters were not expounded upon until Folkerts & Mount (1969; Fig. 1) recognized populations of *G. nigrinoda* inhabiting the lowest reaches of the Mobile Bay Basin, in particular the Tensaw and Mobile rivers, as a distinct subspecies, *Graptemys nigrinoda delticola*. They based the designation on a variety of characters including a dark plastral pattern, various head patterns, soft tissue patterns (predominately black with thin yellow pigmentation lines) and greater carapace height (Fig. 2). By describing *G. n. delticola*, Folkerts & Mount (1969) also erected the name *G. nigrinoda nigrinoda* for specimens with a crescent-shaped or recurved postorbital blotch, dark pigmentation encompassing less than 60% of the plastron and soft

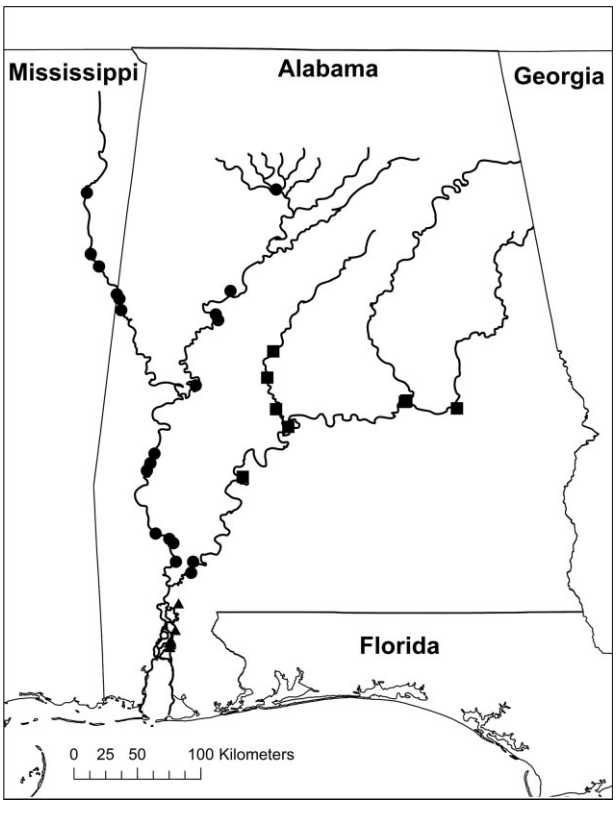

Figure 1. Distribution of the *Graptemys nigrinoda* complex (i.e. *G. n. delticola*, *G. n. nigrinoda* and *G. n. nigrinoda* × *delticola*) museum specimens used in the morphological analyses. Solid triangles represent *G. n. delticola*, solid circles represent *G. n. nigrinoda* × *delticola* and solid squares represent *G. n. nigrinoda*.

Figure 2. Distinguishing characteristics between *Graptemys nigrinoda nigrinoda* and *G. n. delticola*. A, the plastron of *Graptemys n. delticola* (top; male AUM 29434) possesses more dark pigmentation than *G. n. nigrinoda* (bottom; male AUM 06442). B, *Graptemys n. nigrinoda* (right; male AUM 06442) displays more yellow pigmentation on forelimbs (pictured) and other soft tissues than *G. n. delticola* (left; male AUM 29434). C, head patterns between the two subspecies are different in that *G. n. nigrinoda* (left; male AUM 06442) possess more yellow pigmentation and usually has a more crescent-shaped postorbital blotch than *G. n. delticola* (right; male AUM 29434). D, *Graptemys n. delticola* (left; female 8979 AUM) exhibits a more domed carapace than *G. n. nigrinoda* (right; female 10274 AUM).

tissues predominately yellow. These individuals tended to be found in the Alabama (more northern reaches), Cahaba, Coosa and Tallapoosa rivers (but see the distribution of the subspecies in Lahanas, 1986).

The description of *Graptemys nigrinoda delticola* was disputed because of the immense putative hybrid zone between the two subspecies (i.e. Tombigbee and Black Warrior rivers) and lack of an obvious barrier impeding gene flow. Freeman (1970) postulated that the morphological distinctiveness of the Tensaw and Mobile rivers' population was the product of clinal variation. Interestingly, Cagle (1954) had also observed that variation of morphology along the river continuum was evident in other species of turtles inhabiting several coastal rivers, including *G. nigrinoda* (*sensu lato*). However, Freeman's (1970) taxonomic argument against *G. n. delticola* did not gain acceptance in the scientific community (Folkerts & Mount, 1970), and the subspecies remained a recognized taxonomic entity (Ernst & Lovich, 2009; van Dijk *et al*., 2012).

Only one study to date has examined molecular differences between the two subspecies of *G. nigrinoda*. Lamb *et al*. (1994) examined mtDNA restriction site variation as part of a molecular systematic study of the genus *Graptemys*. They found no restriction site differences among five individuals of the two subspecies and that this haplotype was also shared by four other species. In contrast, even with the low levels of mtDNA variation within the genus, sequencing has successfully recovered haplotypes that distinguish closely related *Graptemys* species (e.g. *G. oculifera* and *G. flavimaculata* – Ennen *et al*., 2010a; *G. gibbonsi* and *G. pearlensis* – Ennen *et al*., 2010b).

Due to the lack of standardized criteria for defining subspecies, the subspecies concept has been a highly

debated topic among systematic biologists for over half a century (e.g. Mayr, 1942; Wilson & Brown, 1953). We used both morphological and molecular approaches to investigate the taxonomy of the *Graptemys nigrionda* complex. Often subspecies are described from a limited number of characters; therefore, we examined 47 morphological characters to reassess the taxonomic status of *Graptemys nigrinoda delticola*. We also investigated Freeman's (1970) claim of clinal variation by determining the relationship between morphological variation and environmental factors. Finally, we sequenced a portion of the control region of the mitochondrial genome of several individuals from each subspecies and from the putative intergrade zone.

MATERIALS AND METHODS

Morphometric analyses

Preserved specimens of 78 individuals in the *Graptemys nigrinoda* complex, including individuals identified (by the museums or through geographical location) as *Graptemys nigrionda nigrinoda* (four males, seven females), *G. n. delticola* (six males, 12 females) and *G. n. nigrionda* × *delticola* (38 males, 11 females), were examined from several museums [Auburn University Museum of Natural History and Learning Center (AUM), Carnegie Museum of Natural History (CM) and University of Alabama Museum of Natural History (AL); Appendix]. Morphological characters used in our statistical analyses were selected from Folkerts & Mount's (1969) description of *G. n. delticola* and from a recent study (Ennen *et al*., 2010a) on *G. oculifera* and *G. flavimaculata*. All the characters were measured on the right side of each specimen.

Several quantitative (i.e. continuous variables) features pertaining to the shell and jaw were measured including carapace length (CL), carapace width (CW), carapace height (CH), 1st spine height (SH1; measurement taken from the plastron to the tip of spine), 2nd spine height (SH2; measurement taken from the plastron to the tip of spine), 3rd spine height (SH3; measurement taken from the plastron to the tip of spine), the central seam lengths of the plastron [abdominal length (AB), anal length (AN), femoral length (F), gular length (G), humeral length (H), pectoral length (P)], plastron length (PL), plastron width (PW) and width of jaw (JW; measured from the corners of the tomia). The following quantitative characters were measured based on soft and hard tissue pigmentation using a digital caliper (Mitutoyo 500-196-20): width of yellow and dark pigmentation dorsally (WPIGD and WDPD) and ventrally (WPIGV and WDPV) on the 5th marginal scute, width of the yellow pigmentation on the first vertebral scute (WVPIG), width and length of the yellow blotch on the axial scute (WYAP and LYAP), width and length of the yellow blotch on the inguinal scute (WYIP and LYIP), length and width of interorbital line (LIOL and WIOL), length and width of postorbital blotch (LPOB and WPOB), width of the upper and lower neck lines entering the orbital (WNLO1 and WNLO2), width of dark line between the upper and lower neck lines entering the orbital (WBLO), width of dark pigmentation between the 2nd and 4th lines on the hind limbs (WDH) and forelimbs (WDF), width of 2nd (WY2F) and 4th (WY4F) yellow line on the forelimb, and width of 2nd (WY2H) and 4th (WY4H) yellow line on hind limbs. To quantify the dark pigmentation on the plastron, a key diagnostic feature of *G. nigrinoda delticola* (Folkerts & Mount, 1969), we used a technique similar to that of Lovich, McCoy & Garstka (1990a). A grid of dots 1 cm apart on a clear letter size transparency film (21.59 × 35.56 cm) was created, which was then overlaid on the plastron of each specimen, and the number of dots touching black pigmentation was counted (PLPig).

Several qualitative (i.e. presence/absence and categorical) and meristic characters were measured on each specimen. The following characters were scored as either present or absent: ventral lines connecting under the chin (LLC); a Y pattern created by the two postorbital blotches connecting and extending posteriorly on the neck (Y); interorbital line extending and connecting with lateral line at the nasal (ION); and recurved postorbital blotches. Several meristic characters were also recorded, such as the number of dorsal yellow necklines touching the postorbital blotch (NLPOB), number of lines entering the orbit (NLO), number of digits on the hind limb (YH) and forelimbs (YF) with yellow lines, and number of ventral yellow lines on the forelimb extending to the elbow (YLFE). The categorical characters all related to the classification of the pigmentation pattern on the four pleural scutes. For each pleural scute, whether the yellow pigmentation formed a blotch (0), ring (1) or a broken ring (2) was recorded.

Quantitative and qualitative data were analysed separately. To account for sexual dimorphism (Gibbons & Lovich, 1990), males and females were analysed separately as well. Quantitative variables were standardized for size by CL or PL (only plastron measurements were standardized with PL), and all standardized data were arcsine square root transformed. To summarize the quantitative data, principal components analyses (PCAs) were conducted and loading scores were used to identify important variables driving the morphological gradients. Because environmental data (i.e. current velocity) were shown to shape morphological features of turtle shells

(Rivera, 2008; Rivera & Stayton, 2011; Stayton, 2011), we collected two environmental variables [cumulative drainage area (CDA) and maximum current velocity (MCV); see Schaefer *et al*. (2011)] at every capture point for each specimen to represent the local stream size and hydrology. Both CDA and MCV were collected from the National Hydrology Plus database (http://www.horizon-systems.com/nhdplus/). However, these variables were highly correlated (Pearson's correlations: $r = 0.99$, $P < 0.0001$) for both males and females; therefore, we elected to use CDA as a fixed effect in our analyses. We conducted several multivariate analyses of variance (MANOVAs) to test for differences in the PCA axes scores (1–3) among groups (*G. n. delticola*, *G. n. nigrinoda* and *G. n. nigrindoa* × *delticola*), drainages (body of water in which specimens were captured) and CDA. Pillai values from the MANOVAs have a similar interpretation as R^2 values in multiple regression (Zar, 1999); therefore, we used these values to compare the proportion of the variance in PCA axes 1–3 between our fixed effects.

To analyse the qualitative data, Euclidean distance dissimilarity matrices were created and then used in a non-parametric MANOVA (NP-MANOVA) with group, CDA and drainage as a fixed effects and permutated 10 000 times. All statistical tests were conducted in R statistical software (Vers. 3.0.0, R Development Core Team, Vienna, Austria) with an alpha level of 0.05.

DNA sequence analyses

Tissue samples (tail tips) were collected under the appropriate permits by an author or donated for this project. We extracted total genomic DNA from nine individuals (four *G. n. nigrinoda*, three *G. n. delticola*, two *G. n. nigrinoda* × *delticola*) and sequenced a portion of the mitochondrial control region (CR) using the primers and methods described by Ennen *et al*. (2010). Editing and alignment of the sequence data were conducted in Sequencher v. 4.1. Pairwise uncorrected p-distances between haplotypes were calculated in PAUP* 4.0b10 (Swofford, 2002). TCS v. 1.21 (Clement, Posada & Crandall, 2000) was used to generate a haplotype network based on statistical parsimony TCS with gaps treated as a fifth state. We included control region sequences from *G. flavimaculata* and *G. oculifera* (GenBank accession numbers GQ253568–GQ253571) in the network.

RESULTS

Morphological differentiation

In the PCAs, the first three axes collectively explained 50.2 and 35.4% of the variance in the quantitative morphological characters for females and males, respectively. PCA axis I for males (variance explained: 16.4%) and females (variance explained: 22.0%) produced a pigmentation and carapace-size gradient (Table 1, Figs 3, 4), where *G. n. delticola* possessed more dark pigmentation and higher domed shells. Additionally, PCA axis I for males produced a jaw-width gradient (Fig. 4), where *G. n. delticola* possessed narrower jaws than *G. n. nigrinoda* (Table 2). *Graptemys n. nigrinoda* × *delticola* (i.e. intergrades) possessed intermediate morphologies between *G. n. nigrinoda* and *G. n. delticola* (Table 2, Figs 3, 4).

Table 1. PCA loading scores of female and male *Graptemys nigrinonda nigrinoda*, *G. n. delticola* and *G. n. nigrinoda* × *delticola* of several characters driving the gradients along axis I; percentages in parentheses represent variance explained by axes I, II and III, respectively

Characters/sex	Axis I	Axis II	Axis III
Female (22.0%, 18.7%, 9.4%)			
CH/CL	−0.258	−0.232	0.035
3rd Spine/CL	−0.230	−0.240	0.134
LOPB/CL	0.203	0.019	0.138
LYAP/CL	0.200	0.166	−0.059
WY2F/CL	0.281	−0.157	0.018
WY4F/CL	0.238	−0.191	0.024
WB24F/CL	−0.278	−0.042	0.003
WY2H/CL	0.208	−0.123	−0.127
WB24H/CL	−0.276	−0.069	−0.160
PLPig	−0.316	−0.015	−0.073
Male (16.4%, 10.1%, 8.9%)			
CW/CL	−0.288	0.055	−0.038
JW/CL	−0.319	0.017	−0.032
1st Spine/CL	−0.264	−0.296	0.091
2nd Spine/CL	−0.227	−0.191	0.229
WNLO2/CL	−0.295	0.021	0.038
WY4F/CL	−0.211	0.178	0.070
WIOL/CL	−0.254	−0.055	−0.102
LYIP/CL	−0.204	0.060	0.033
WYIP/CL	−0.264	0.107	0.025

For the quantitative data, there were significant differences in morphology among specimens (Table 3). In females, there were differences in morphology among groups ($F_{2,17} = 10.24$, $P < 0.001$, Pillai = 1.32) and drainages ($F_{3,17} = 2.60$, $P = 0.015$, Pillai = 0.94). However, morphological differences were also attributed to CDA ($F_{1,17} = 9.03$, $P = 0.001$, Pillai = 0.64). In males, there were also significant differences in morphology among groups ($F_{2,36} = 4.38$, $P = 0.001$, Pillai = 0.55) and drainages ($F_{3,36} = 2.33$, $P = 0.019$, Pillai = 0.49). Similar to females, male morphology was related to CDA ($F_{1,36} = 3.72$, $P = 0.021$, Pillai = 0.25). Also, there were significant interactions between CDA and both group ($F_{2,36} = 3.95$, $P = 0.002$,

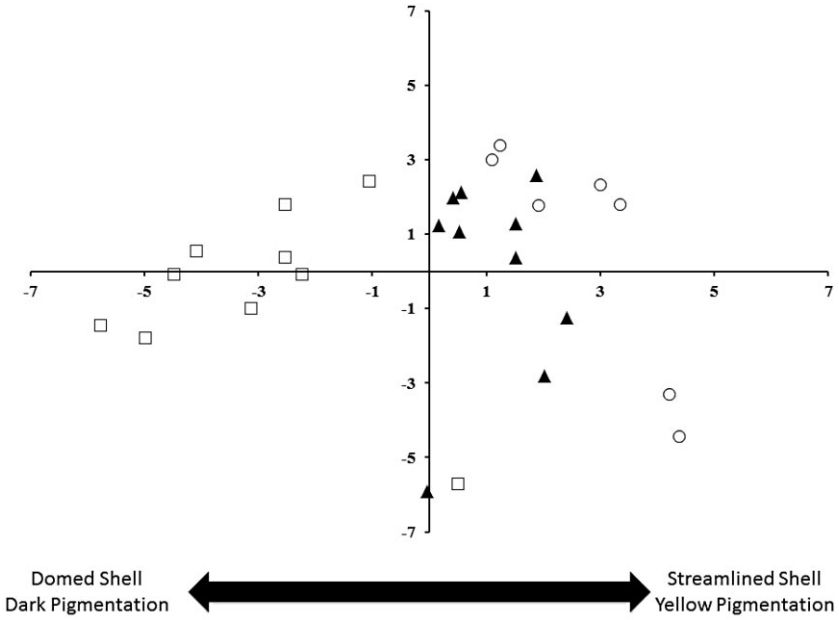

Figure 3. A PCA plot of female specimens displaying a gradient of the pigmentation and carapace height along Axis I. Open circles represent *Graptemys nigrinoda delticola* specimens, open squares represent *G. nigrinoda nigrinoda* specimens and solid triangles represent *Graptemys n. nigrinoda* × *delticola.*

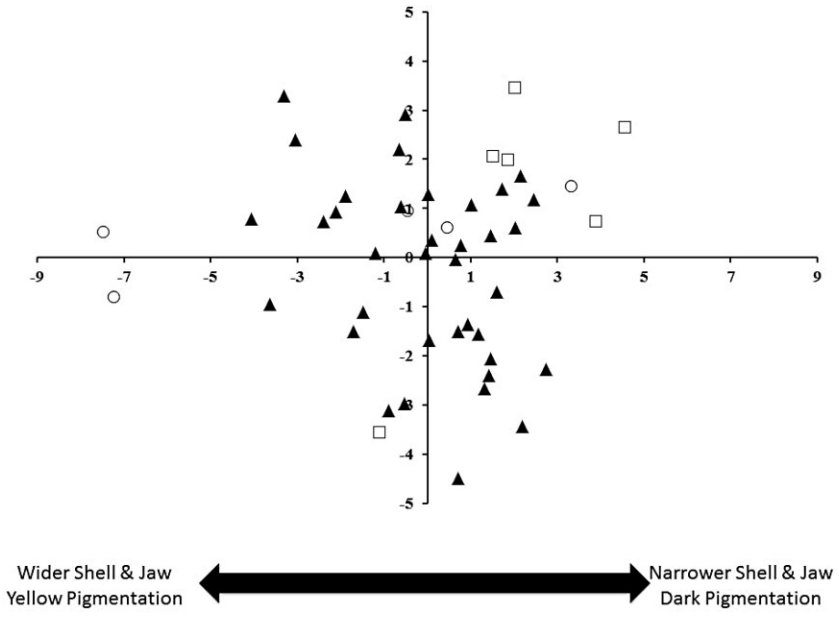

Figure 4. A PCA plot of male specimens displaying a gradient of the pigmentation and carapace height along Axis I. Open circles represent *Graptemys nigrinoda delticola* specimens, open squares represent *G. nigrinoda nigrinoda* specimens and solid triangles represent *Graptemys n. nigrinoda* × *delticola.*

Pillai = 0.51) and drainage ($F_{3,36}$ = 3.63, P = 0.001, Pillai = 0.70). The group effect had the highest Pillai value in females (1.32). However, the interaction between CDA and drainage showed the highest Pillai value in males (0.70).

Because there was an interaction between group and CDA in males and to further investigate the clinal variation claim of Freeman (1970), we conducted separate MANOVAs on *G. n. nigrinoda* × *delticola* using CDA and drainage as effects. *Graptemys n. nigrinoda* ×

Table 2. Mean ratios and standard deviation (SD) of several (but not all) important pigmentation characters for females and/or males determined by the PCA loading scores that can differentiate between *Graptemys n. nigrinoda*, *Graptemys n. nigrinoda* × *delticola* and *Graptemys n. delticola* in the Mobile Bay Basin drainage

	Shell variables			Yellow pigmentation					Dark pigmentation	
Group/sex	CW/CL	CH/CL	JW/CL	LOPB/CL	WIOL/CL	WY2F/CL	WY4F/CL	WY2H/CL	WDF/CL	WDH/CL
Graptemys n. nigrinoda										
Male										
Mean	1.024	0.146	0.018	5.44E-03	3.33E-04	8.27E-04	3.61E-04	4.09E-04	1.37E-03	1.56E-03
SD	0.273	0.030	0.010	1.62E-03	3.35E-04	1.72E-04	6.34E-05	5.57E-05	3.87E-04	5.64E-04
Female										
Mean	0.859	0.147	0.014	2.36E-03	1.10E-04	5.10E-04	1.76E-04	2.17E-03	1.54E-03	2.15E-03
SD	0.067	0.014	0.001	1.40E-03	5.00E-05	2.86E-04	8.55E-05	1.45E-04	2.33E-04	6.78E-04
Graptemys n. nigrinoda × *delticola*										
Male										
Mean	0.929	0.15	0.015	6.74E-03	1.84E-04	5.51E-04	2.56E-04	2.28E-04	1.60E-03	1.78E-03
SD	0.151	0.024	0.002	5.57E-03	1.07E-04	1.70E-04	1.22E-04	9.34E-05	4.86E-04	7.36E-04
Female										
Mean	0.952	0.156	0.014	2.30E-03	1.20E-04	3.98E-04	1.71E-04	1.80E-04	1.80E-03	2.33E-03
SD	0.171	0.021	0.002	1.13E-03	8.16E-05	1.20E-04	8.04E-05	7.04E-05	3.18E-04	4.17E-04
Graptemys n. delticola										
Male										
Mean	0.823	0.151	0.011	4.40E-03	7.93E-04	2.81E-04	1.53E-04	1.57E-04	2.34E-03	3.36E-03
SD	0.066	0.009	0.001	3.71E-03	1.57E-04	1.37E-04	4.56E-05	5.63E-05	9.02E-04	6.95E-04
Female										
Mean	0.820	0.198	0.013	1.02E-03	6.80E-04	1.53E-04	8.95E-05	1.24E-04	2.34E-04	3.77E-03
SD	0.060	0.023	0.001	5.82E-04	3.39E-05	8.57E-05	7.46E-05	6.34E-05	6.94E-04	1.05E-03

Table 3. Result of the quantitative data using MANOVAs for female and male specimens of *Graptemys nigrinoda*

	d.f.	Pillai	*F*	*P*
Female				
CDA	1	0.64	9.03	0.001
Group	2	1.32	10.24	< 0.001
Drainage	3	0.94	2.60	0.015
CDA × Group	2	0.18	0.53	0.778
CDA × Drainage	1	0.31	2.27	0.122
Residuals	17			
Male				
CDA	1	0.25	3.72	0.021
Group	2	0.55	4.38	0.001
Drainage	3	0.49	2.33	0.019
CDA × Group	2	0.51	3.95	0.002
CDA × Drainage	3	0.70	3.63	0.001
Residuals	36			

delticola specimens were chosen due to low sample sizes in the other two groups. For female *G. n. nigrinoda* × *delticola*, CDA was related to morphology ($F_{1,6} = 7.79$, $P = 0.038$, Pillai = 0.85) and morphological differences were found among drainages ($F_{1,6} = 6.65$, $P = 0.049$, Pillai = 0.83). For male *G. n. nigrinoda* × *delticola*, morphological differences occurred among drainages ($F_{2,31} = 3.57$, $P = 0.004$, Pillai = 0.52); however, CDA ($F_{1,31} = 2.62$, $P = 0.07$, Pillai = 0.21) and the interaction between CDA and drainage ($F_{2,31} = 2.11$, $P = 0.07$, Pillai = 0.35) were both close to significant.

For the qualitative data, there were also significant differences in morphology among specimens. In females, there were significant differences among groups ($F_{2,28} = 3.18$, $P = 0.024$), and morphology was related to CDA ($F_{1,28} = 11.67$, $P < 0.001$). There was a significant interaction between the group and CDA variables ($F_{2,28} = 3.00$, $P = 0.018$), which might be interpreted as representing clinal variation in these characters. In males, there were significant differences in morphology among drainages ($F_{4,46} = 4.62$, $P < 0.001$) but not among the groups or related to CDA (Table 4).

Genetic differentiation

Five unique haplotypes (657–658 bp; GenBank accession numbers KF494953–KF494957) were found in the nine control region sequences from the *G. nigrinoda delticola*, *G. n. nigrinoda* × *delticola* and *G. n. nigrinoda* individuals. Only 1–2 base substitutions were seen among these haplotypes (0.15–0.30% uncorrected p-distance). One haplotype was found in both subspecies and the intergrades while three haplotypes were found only in *G. n. nigrinoda* (Table 5; Fig. 4). One *G. oculifera* haplotype was only two mutational steps from the *G. nigrinoda* portion of the network, but a large phylogenetic break separated the remaining *G. flavimaculata* and *G. oculifera* haplotypes (Fig. 4).

DISCUSSION

Subspecies descriptions based on morphological characters, especially those under strong selection, may be incongruent with evolutionary history (see Burbrink, 2000; Burbrink *et al*., 2000). Given this potential incongruence, testing subspecies designations via phylogeographical analyses is becoming commonplace (Burbrink *et al*., 2000; Mulcahy, 2008). In our analyses of *Graptemys nigrinoda* (complex), we found that the taxonomy based on morphological characteristics does not reflect any underlying phylogenetic structure in the group, but instead the morphological differences among the groups seem to represent clinal variation along an environmental gradient.

The molecular data did not provide evidence for the validity of the two subspecies, a result that is congruent with an earlier study (Lamb *et al*., 1994). Limited sequence divergence distinguished the haplotypes, of which one was shared by the subspecies (Fig. 5). Admittedly, mtDNA is not particularly variable in turtles, but it has been used successfully to address taxonomic issues in other species of *Graptemys* (Lamb *et al*., 1994; Ennen *et al*., 2010a, b) and other turtle species (Fitzsimmons & Hart, 2007). The disparate results between the morphological and molecular data are not uncommon within the genus *Graptemys* (Ennen *et al*., 2010a, b) but not necessarily typical (Lovich & McCoy, 1992; Lamb *et al*., 1994). Any number of factors may lead to morphological differentiation in the absence of strong genetic differentiation, such as lineage sorting of polymorphism, environmental effects or natural selection (Ennen *et al*., 2010a).

While there is no geographical pattern of genetic differentiation, there is morphological variation within the *Graptemys nigrinoda* complex, but it is questionable whether this is worthy of taxonomic recognition. The MANOVAs found significant differences among the three groups for both male and female specimens with the exception of males in the qualitative analysis. However, the MANOVAs also found significant differences based on the environmental effects (i.e. CDA, drainage, and/or the interaction terms between CDA and group), suggesting that some of the morphological variation may be associated with a cline or a particular habitat. The PCA also illustrates a clear morphological gradient as both carapace height and pigmentation separate the groups along axis 1 (Figs 2, 3). The two subspecies are found at the extremes of the gradient, and the

Table 4. Result of the qualitative data using NP-MANOVAs for female and male specimens of *Graptemys nigrinoda*

	d.f.	SS	*F*	R^2	*P*
Female					
CDA	1	69.872	11.673	0.248	< 0.001
Group	2	38.13	3.185	0.136	0.024
Drainage	1	4.82	0.805	0.017	0.425
CDA:Group	2	35.953	3.003	0.128	0.018
CDA:Drainage	1	6.896	1.152	0.025	0.313
Residuals	21	125.708		0.447	
Male					
CDA	1	12.686	2.480	0.041	0.086
Group	1	5.5	1.075	0.018	0.319
Drainage	4	94.522	4.619	0.306	< 0.001
CDA:Drainage	4	15.229	0.744	0.049	0.657
CDA:Group	1	1.854	0.363	0.006	0.737
Residuals	35	179.06		0.580	

Table 5. Locations (i.e. Drainage and GPS coordinates) and number of control region sequences (*N*) obtained for each group within the *Graptemys nigrinoda* complex; the frequency of each of the five unique haplotypes in each drainage is also indicated

			Haplotype frequency					GPS coordinates	
Group	Drainage	*N*	1	2	3	4	5	Latitude	Longitude
G. n. nigrinoda	Cahaba	2	1	–	–	–	1	32.320417	–87.093450
G. n. nigrinoda	Tallapoosa	2	–	–	1	1	–	32.500030	–86.254230
G. n. nigrinoda × *delticola*	Tombigbee	2	1	1	–	–	–	32.061200	–88.110767
G. n. delticola	Tensaw	3	2	1	–	–	–	30.798081	–87.920609

putative intergrade specimens are distributed in between the two subspecies. This pattern could be the product of clinal variation rather than hybridization. In situations such as this, the growing consensus is that these subspecies names should be synonymized (Manier, 2004; Mulcahy, 2008).

Rivers are typically characterized by changes in their physical parameters as they progress from headwaters to their mouth (i.e. river continuum concept; Vannote *et al*., 1980). Width, turbidity, discharge and temperature usually increase along the gradient of the river continuum, with headwaters characterized by faster flowing (higher gradients), clear and cool water to the lower stream reaches characterized by slow-flowing (low gradients), turbid and warm habitats. The distribution of *G. nigrinoda* spans a large portion of the river continuum in the Mobile Bay Basin, which is the largest drainage system east of the Mississippi River that empties into the Gulf of Mexico (USACE, 1985). The large size and complex physiography of the Mobile Bay Basin producing a range of environmental conditions and selective forces have probably contributed to the presence of numerous endemic species (Lydeard & Mayden, 1994).

Morphological variation along the river continuum has not been well studied in *Graptemys* nor turtles in general. However, differences in body shape (i.e. carapace height-to-length, carapace width-to-length and head width-to-length ratios) were shown within *G. flavimaculata* between two sites along the river continuum but not a third (Selman, 2012). He postulated that the body-shape patterns could be attributed to environmental and biotic factors (i.e. food resources, competition, environmental variables and the presence of the predator *Alligator mississippiensis*). Our carapace height-to-length and width-to-length gradient pattern was very similar to that found by Selman (2012). Environmental factors, such as water velocity, are known to influence body shape of turtles, as several studies have reported shape differences between turtles inhabiting lentic (i.e. low water velocity) and lotic (i.e. high water velocity) sites (Aresco & Dobie, 2000; Lubcke & Wilson, 2007). Rivera (2008) found that body shape, in particular carapace

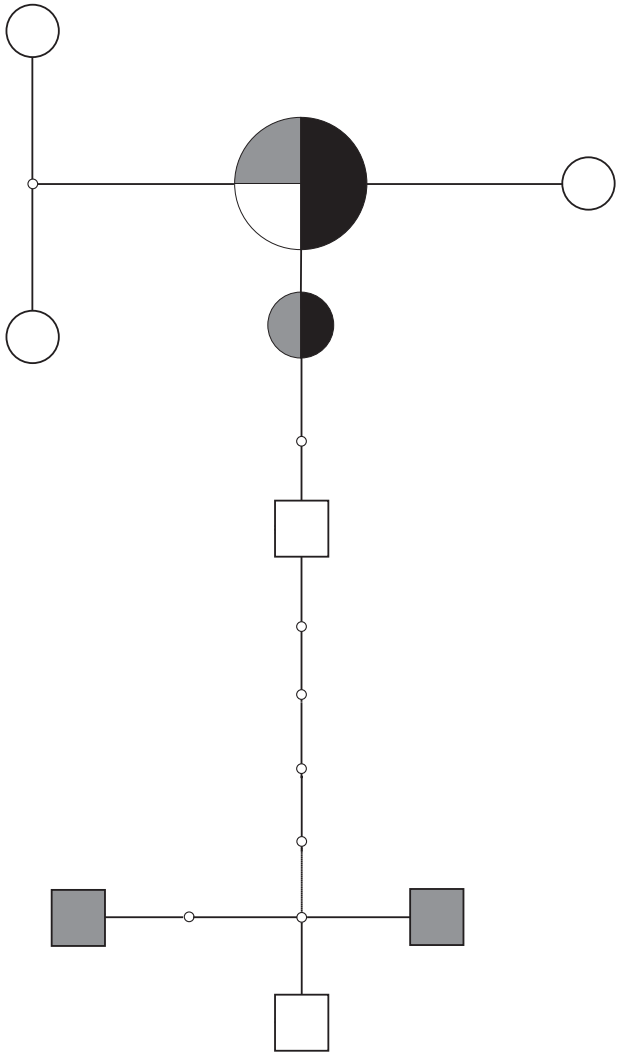

Figure 5. The haplotype network for the mitochondrial control region sequences for *Graptemys nigrinoda* subspecies (circles), *G. flavimaculata* (squares with grey fill) and *G. oculifera* (squares with no fill). The frequency of the haplotypes for *G. nigrinoda* is indicated by the size of the circle and the colour indicates the location of the haplotype as follows: Tensaw, black; Tallapoosa and Cahaba, white; Tombigbee, grey.

height-to-length, of *Pseudemys concinna* differed significantly between lentic and lotic systems and he also provided empirical evidence that more streamlined individuals were selected for in lotic systems because of drag reduction.

Pigmentation, such as head patterns, in other *Graptemys* species has been shown to demonstrate clinal variation, presumably influenced by temperature (Ewert, 1979; Vogt, 1993). Interestingly, the important loading scores in our PCA included three head pigmentation patterns (i.e. LOPB, WNLO2 and WIOL); however, most of the pigmentation gradients were driven by other soft-tissue pigmentation (i.e. hind limbs and forelimbs). The relationship between temperature and soft-tissue pigmentations has not been studied to date within *Graptemys*, mainly because these features were not considered taxonomically useful until recently (Ennen *et al*., 2010a). Therefore, more investigations are warranted on the influence of incubation temperatures on soft-tissue pigmentation patterns, in particular on appendages, within *Graptemys*.

Soft-tissue pigmentation could also be driven by natural selection in which darker pigmentation is favoured in more turbid water and yellow pigmentation is favoured in clearer water. For example, many fish species evolved colour vision to increase the contrast of prey from their background (McFarland & Munz, 1975; Ohguchi, 1981) and subsequent co-evolution causes prey items to become more cryptic over time. The pigmentation cline could also be attributed to differing female preferences for male pigmentation patterns depending on the level of turbidity. Although female preference in male attributes is unknown in *G. nigrinoda* (e.g. Lovich, Garstka & Cooper, 1990b), fish mating colours, which are strongly related to attraction, become melanistic in turbid water (McDonald, Reimchen & Hawryshyn, 1995).

Male specimens displayed a jaw width cline along the river continuum as well, where specimens near the mouth (i.e. Tensaw River) possessed narrower jaws. A species' jaw morphology and associated muscles are intrinsically linked to diet (Tucker, Fitzsimmons & Gibbons, 1995; Tucker, Yeomans & Gibbons, 1997; Pfaller, Gignac & Erickson, 2011). This association can produce distinct intraspecific morphologies referred to as resource use polymorphism within other taxa (e.g. fish, amphibians, and birds; Smith & Skúlason, 1996). Dietary studies on *Graptemys nigrinoda* are limited, the only study being by Lahanas (1986), who focused on *G. n delticola*; therefore, resource use polymorphism within the species cannot be assessed without additional research on diet. However, the wider jaw width in males could be associated with a dietary shift to larger or harder prey items along the river continuum. For example, some mussels and gastropods display phenotypic variation in shell smoothness and width (inflated or not) along the river continuum, where individuals upstream are less inflated and smoother (Minton *et al*., 2008). If this clinal variation phenomenon occurs in molluscs in the Mobile Bay Basin, the reduction of width of molluscs such as snails upstream could allow male *Graptemys nigrinoda* (*sensu lato*) to consume this prey item in greater frequency, potentially selecting for larger jaws capable of crushing the prey. However, dietary

samples from the Alabama River in Autauga and Lowndes counties contain no snails but some small bivalves (P. Lindeman pers. comm.); therefore, further dietary studies along the river continuum are needed.

The broad geographical range of the intergrades is another troubling aspect of the presumed distribution of the subspecies within the *Graptemys nigrinoda* complex. *Graptemys nigrinoda nigrinoda* is found in the upper drainages of the Mobile Bay Basin such as the Alabama, Cahaba, Coosa, and Tallapoosa rivers, while *G. n. delticola* is found in the extreme lower portion of the Mobile Bay Basin, in the Mobile and Tensaw rivers. According to Lahanas (1986) and Mount (1975), intergrades are then found everywhere else, including all of the Tombigbee, Black Warrior and lower Alabama (below the Wilcox–Monroe County line) rivers, and even in the region of the Black Warrior River where the type specimen of *Graptemys nigrinoda* was collected. The range of the intergrades is greater than either of the two subspecies. This pattern is highly unusual, as the literature on hybridization shows that most hybrid zones make up only a relatively narrow portion of the species' range (Barton & Hewitt, 1989).

It appears that the features used to delineate subspecies within *Graptemys nigrinoda* were not phylogenetically relevant but rather represented geographical variation along a cline. Historically, *Graptemys* systematics and taxonomy were highly reliant on colour patterns on the shell and head (Lovich & McCoy, 1992; Vogt, 1993; Ennen *et al*., 2010a, b); however, the use of colour pattern characteristics for delineating subspecies may not always reflect the underlying phylogenetic relationships among groups due to strong selection pressures (e.g. Burbrink *et al*., 2000; Manier, 2004). Because of the association of morphological variation with environmental variables, the absence of distinct molecular differences, and a range that lacks any strong barriers to gene flow between the subspecies, we recommend that *G. n. delticola* should not be retained as a valid taxonomic entity.

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APPENDIX

Graptemys nigrinoda – Alabama River: AUM5948, AUM6308, AUM12553, AUM22911; Black Warrior River: AL72-175, AL72-176, AUM10144, AUM10149, AUM12628, AUM12630, AUM12631, AUM12632, AUM12633, AUM12634, AUM12635, AUM12636, AUM12638, AUM12639, AUM12640, AUM12641, AUM12642, AUM18418, AUM18419, AUM18420, AUM18423, AUM18425; Cahaba River: AUM9263, AUM9267, AUM10110, AUM27949; Coosa River: AUM5965; Tallapoosa River: AUM9157, AUM10274, AUM11816, AUM38922, AUM38965; Tensaw River: AUM8968, AUM8973, AUM10716, AUM28193, AUM28194, AUM28195, AUM28207, AUM28208, AUM28682, AUM28790, AUM28792, AUM29258, AUM29435, AUM29471, CM95911, CM95909, CM95910; Tombigbee River: AL72-6, AL72-7, AL72-72, AL72-73, AL72-91, AL72-93, AL72-98, AL72-99, AL72-100, AL72-101, AUM6304, AUM8789, AUM8791, AUM9254, AUM9272, AUM9345, AUM10301, AUM10302, AUM10303, AUM12557, AUM12689, AUM12856, AUM17132.