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Author(s): James C. Godwin, Jeffrey E. Lovich, Joshua R. Ennen, Brian R. Kreiser, Brian Folt, and Chris Lechowicz
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Hybridization of Two Megacephalic Map Turtles (Testudines: Emydidae: *Graptemys*) in the Choctawhatchee River Drainage of Alabama and Florida

James C. Godwin[1], Jeffrey E. Lovich[2], Joshua R. Ennen[3], Brian R. Kreiser[4], Brian Folt[5], and Chris Lechowicz[6]

Map turtles of the genus *Graptemys* are highly aquatic and rarely undergo terrestrial movements, and limited dispersal among drainages has been hypothesized to drive drainage-specific endemism and high species richness of this group in the southeastern United States. Until recently, two members of the megacephalic ''*pulchra* clade,'' *Graptemys barbouri* and *Graptemys ernsti*, were presumed to be allopatric with a gap in both species' ranges in the Choctawhatchee River drainage. In this paper, we analyzed variation in morphology (head and shell patterns) and genetics (mitochondrial DNA and microsatellite loci) from *G. barbouri*, *G. ernsti*, and *Graptemys* sp. collected from the Choctawhatchee River drainage, and we document the syntopic occurrence of those species and back-crossed individuals of mixed ancestry in the Choctawhatchee River drainage. Our results provide a first counter-example to the pattern of drainage-specific endemism in megacephalic *Graptemys*. Geologic events associated with Pliocene and Pleistocene sea level fluctuations and the existence of paleo-river systems appear to have allowed the invasion of the Choctawhatchee system by these species, and the subsequent introgression likely predates any potential human-mediated introduction.

THE southeastern United States is globally important as a region characterized by high aquatic biodiversity (Lydeard and Mayden, 1995). For example, freshwater turtle species richness is notably high, second only to that in the Ghanges-Brahmaputra River basin in Southeast Asia (Buhlmann et al., 2009). Many of the turtle species in the southeastern U.S. are sympatric, providing at least the potential for hybridization and introgression. Of the entire freshwater turtle fauna in the southeastern United States, the genus *Graptemys* is the most species rich with 13 described species (Ernst and Lovich, 2009; Ennen et al., 2010a). Species of *Graptemys* possess highly aquatic habits (i.e., terrestrial movements are restricted to nesting females; Shealy, 1976), a behavior that is hypothesized to limit dispersal among drainages and drive drainage-specific endemism in this group (Lamb et al., 1994). Consequently, along the Gulf Coast of the U.S., the genus *Graptemys* exhibits distributional and endemism patterns paralleling those of other aquatic species, such as fish and freshwater mussels (Lydeard and Mayden, 1995; Walker and Avise, 1998; Boschung and Mayden, 2004; Williams et al., 2008).

All five species of *Graptemys* within the *pulchra* clade (i.e., *G. pulchra*, *G. barbouri*, *G. ernsti*, *G. gibbonsi*, and *G. pearlensis*; *sensu* Lamb et al., 1994) generally exhibit drainage-specific endemism (with the minor exception of *G. ernsti* in both the Escambia and Yellow rivers that drain into a common estuary). However, the discovery of both putative individuals of *G. barbouri* and *G. ernsti* in the Choctawhatchee River in the 1960s (i.e., unpublished accounts but vouchered specimens in Auburn University Museum of Natural History) and 1990s (Godwin, 2004; Enge and Wallace, 2008; Ernst and Lovich, 2009; Lovich et al., 2011) challenge the drainage-specific axiom for the group. Typically, *G. barbouri* is considered to be restricted to the Apalachicola River drainage, whereas *G. ernsti* is restricted to the Conecuh-Escambia and Yellow River systems (hereafter Escambia-Yellow system; Fig. 1). These drainages are located to the east and west of the Choctawhatchee-Pea drainage of Alabama and Florida. The *Graptemys* inhabiting the Choctawhatchee-Pea drainage were tentatively identified as *G. barbouri*, *G. ernsti*, or putative hybrids, because some individuals possess patterns intermediate between those of the two neighboring species (JCG and JEL, pers. obs.). In addition, *Graptemys* have also been recently recorded from the Wacissa (Jackson, 2003) and Ocklockonee (Enge and Wallace, 2008) rivers to the east of the Apalachicola River (Fig. 1). In both cases of these new finds, it is unknown if these populations are natural or a result of human translocation (Jackson, 2005). If in fact both *G. barbouri* and *G. ernsti* naturally occur within the Choctawhatchee and Pea rivers, this would be the first record of the syntopy of two megacephalic species of *Graptemys* (Lindeman, 2000). However, no study has rigorously investigated the identity of *Graptemys* in the Choctawhatchee and Pea rivers.

Graptemys barbouri and *G. ernsti* have been considered distinct, and until now, allopatric species since Lovich and McCoy (1992) first reviewed the taxonomy of the *G. pulchra* clade and described *G. ernsti*. The monophyly of the *pulchra* clade was strongly supported by mtDNA phylogenies (Lamb et al., 1994). In addition to statistically significant morphological differences in the relative lengths of their paired major plastron scutes, *G. barbouri* and *G. ernsti* differ consistently in upper and lower marginal pigmentation and head patterns. In addition, the mtDNA data of Lamb et al. (1994) were congruent with the morphological and pattern-based analyses of Lovich and McCoy in supporting recognition of *G. gibbonsi* (*sensu lato*, Ennen et al., 2010a) and *G. ernsti* as discrete taxa. With the exception of Artner

[1] Alabama Natural Heritage Program, Auburn University Museum of Natural History, 1090 S. Donahue Drive, Auburn University, Auburn, Alabama 36849; E-mail: jcg0001@auburn.edu. Send reprint requests to this address.
[2] U.S. Geological Survey, Southwest Biological Science Center, 2255 North Gemini Drive, MS-9394, Flagstaff, Arizona 86001; E-mail: jeffrey_lovich@usgs.gov.
[3] Tennessee Aquarium Conservation Institute, One Broad Street, Chattanooga, Tennessee 37402; E-mail: jre@tnaqua.org.
[4] Department of Biological Sciences, University of Southern Mississippi, Hattiesburg, Mississippi 39406; E-mail: brian.kreiser@usm.edu.
[5] Department of Biological Sciences and Auburn University Museum of Natural History, 331 Funchess Hall, Auburn University, Auburn, Alabama 36849; E-mail: brian.folt@gmail.com.
[6] Sanibel Captiva Conservation Foundation, P.O. Box 839, 3333 Sanibel-Captiva Road, Sanibel, Florida 33957; E-mail: clechowicz@sccf.org.
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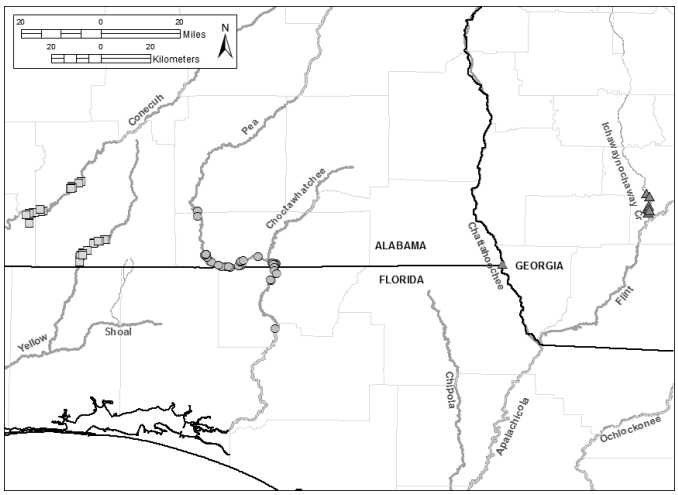

Fig. 1. Gulf Coastal Plain river systems of Alabama, Florida, and Georgia from which taxa of *Graptemys* were collected for the taxonomic assessment of the Choctawhatchee and Pea river population. Open squares represent collection localities for *Graptemys ernsti* from the Conecuh and Yellow rivers, Alabama; open triangles represent collection localities for *Graptemys barbouri* from Ichawaynochaway Creek, Georgia; solid diamonds represent collection localities for *Graptemys* sp. in the Choctawhatchee and Pea rivers, Alabama and Florida.

(2008), who proclaimed that *G. barbouri* and *G. ernsti* were subspecies of *G. pulchra* with no analyses to support the claim, modern treatments of the genus have not questioned the specific status of *G. ernsti* and *G. barbouri* (e.g., Bonin et al., 2006; Fritz and Havaš, 2007; Ernst and Lovich, 2009; van Dijk et al., 2012; Lindeman, 2013).

The distribution of species of *Graptemys* strongly reflects historical and contemporary competitive interactions (Lindeman, 2000). Adaptive radiation in the genus has produced two functional trophic groups morphologically characterized by microcephalic and megacephalic head shapes. The evolution of head-size differences was long postulated to reduce intra- and interspecific competition through resource partitioning between species of *Graptemys* and the sexes (Lindeman, 2000). Megacephaly is expressed only in females of the *pulchra* clade, and intraspecific intersexual resource competition is hypothesized to have driven character displacement in this clade. Until the reports of individuals of *G. barbouri* and *G. ernsti* from the Choctawhatchee drainage, megacephalic species typically only occurred sympatrically with microcephalic congeners, which occur in unique combinations according to drainage (Lindeman, 2000; Ernst and Lovich, 2009). One exception to this pattern exists in the upper reaches of the Coosa and Cahaba rivers of Alabama, where *G. pulchra* co-occurs with the mesocephalic *G. geographica* (Mount, 1975). However, the putative discovery of sympatric megacephalic species in the Choctawhatchee and Pea rivers (*G. barbouri* and *G. ernsti*) ostensibly provides an example where two species of *Graptemys* with functionally identical feeding niches co-occur, challenging the paradigm of competition and drainage-specific endemism in the biogeography of the genus.

In this study, we sought to identify the megacephalic *Graptemys* inhabiting the Choctawhatchee and Pea rivers using a comparative framework. We used morphological and genetic techniques to compare individuals collected from the Choctawhatchee and Pea rivers to *Graptemys barbouri* and *Graptemys ernsti* individuals collected from the cores of their respective known ranges. Because *Graptemys* are known to hybridize (Freedberg and Myers, 2012), we also assessed the putative introgression between *G. barbouri* and *G. ernsti* within the Choctawhatchee and Pea rivers suggested by Godwin (2004).

MATERIALS AND METHODS

Sampling methods.—We collected *Graptemys* spp. from the mainstem of the Choctawhatchee and Pea rivers, Geneva County, Alabama and Holmes County, Florida. The Choctawhatchee River watershed is confined entirely to the Coastal Plain physiographic province, draining 9,417 km^2 of land (Witmer et al., 2009). For comparative purposes, *G. barbouri* and *G. ernsti* were collected from representative populations in the cores of their ranges: *G. barbouri* were collected from Ichawaynochaway Creek, a tributary of the Flint River (Apalachicola drainage) in Georgia, and *G. ernsti* were collected in the Conecuh and Yellow rivers, the upper reaches of the Escambia-Yellow system, in Alabama (Fig. 1).

We captured turtles by hand and with basking traps from April to August 2012. We recorded the date and locality of each capture using a handheld GPS unit (decimal degrees, WGS 84). Turtles were measured for shell and pattern variables (±0.1 mm; see below) and mass (±1 g). Sex was identified for adult turtles by assessing post-cloacal tail length. Tissue samples were collected from each individual (e.g., tail tip) and stored in 95% ethanol. Individuals were uniquely marked with a combination of marginal scute notches to facilitate identification upon recapture to prevent repeated sampling. Once data and tissue samples were obtained, most turtles were released, except for a series vouchered in the collection of the Auburn University Museum of Natural History (n = 4). Measurements and/or tissues were collected from a total of 146 specimens across five stream reaches (Table 1).

Categorical head variables.—Qualitative data were collected based on six head patterns that discriminate *G. barbouri* and *G. ernsti* (Lovich and McCoy, 1992; Ernst and Lovich, 2009; Table 2). For three variables, individuals were scored as possessing one of three character states: POB-IOB and chinbar were scored as complete, intermediate, or absent, and the nasal pattern was scored as being a trident, an arrow, or an intermediate form. Three additional characters (SUPOC, SUBOC, and dorsal heart-shaped head blotch) were scored as present/absent. We analyzed these qualitative traits in two ways. First, we used contingency table analyses to generate Pearson's Chi-square values to compare head patterns of *Graptemys* from the Choctawhatchee River drainage to known *G. barbouri* and *G. ernsti* to test whether or not *Graptemys* in the Choctawhatchee differ in the frequency of these variables relative to *G. barbouri* and *G. ernsti* from within their known ranges. This was done by using Pearson's Chi-square test to compare the frequencies of character states from the Choctawhatchee drainage directly to those in the Apalachicola drainage (*G. barbouri*) and Escambia-Yellow system (*G. ernsti*). The *P*-values of these pair-wise tests were tested for significance with sequential Bonferroni correction (Rice, 1989) to account for multiple comparisons. We did not run tests comparing *G. barbouri* and *G. ernsti*, because the variables of interest are known to differ between these taxa, and their taxonomic distinctness is not in question (Ernst and Lovich, 2009; van

Table 1. Number of male (M) and female (F) *Graptemys barbouri*, *Graptemys ernsti*, and *Graptemys* spp. collected from south Alabama, Florida, and Georgia river systems for discriminant function morphometric analyses, and the total number of tissue samples from each river that were used for molecular analyses. The Ichawaynochaway sample included one male museum specimen from the Chattahoochee River.

Species	River	Morphometrics		Tissue samples
		F	M	
Graptemys barbouri	Ichawaynochaway (Flint)	26	1	30
Graptemys ernsti	Conecuh	21	6	35
Graptemys ernsti	Yellow	5	9	26
Graptemys sp.	Choctawhatchee	2	2	14
Graptemys sp.	Pea	5	8	29

Dijk et al., 2012). Secondly, we calculated a morphological hybrid index (MHI) modified from Heiser (1949) and Stebbins and Daly (1961). For each specimen, we scored each morphological trait on a scale of 0–2 (0 = *G. barbouri*, 1 = intermediate, 2 = *G. ernsti*), and then calculated the average score across the measured traits. Because we were collecting data from live specimens and some individuals would retract their heads into their shells, in some instances we could not record all six morphological traits for an individual. Therefore, we used only individuals from which we could record data for at least four morphological traits. We conducted a Spearman's correlation analysis using MHI average score and q scores from STRUCTURE based on each individual's genetic data (see below for explanation of genetic methodology) to determine the relationship between morphology and genetic data. We tested whether MHI scores differed among all drainage systems and whether scores differed between the Choctawhatchee and Pea rivers using a Kruskal-Wallis test and a Wilcoxon signed-rank test, respectively. Chi-square, Spearman, Kruskal-Wallis, and Wilcoxon analyses were performed in the statistical program R (R Development Core Team, 2012).

Morphometrics.—Besides the six qualitative traits, we measured several quantitative variables found useful by Lovich and McCoy (1992) and conducted a discriminant function analysis. All measurements were collected by a single researcher (JCG) on the right side of each individual. Morphometric data collected from individuals included measurements of the fifth marginal scute width (MWID), dorsal and ventral pigmentation patterns on the fifth marginal scute (i.e., width of light colored pigment on dorsal surface [MPIG] and width of dark pigment on ventral surface [MLWP]), and the length of the post-orbital blotch (LPOB). Mensural shell variables included carapace height and width along with midline length, and six pairs of plastron scutes. Plastron scute measurements have been used in previous studies to measure degree of similarity between and among turtle taxa (Lovich and Ernst, 1989; Lovich et al., 1991; Ernst et al., 1997), including *Graptemys* (Lovich and McCoy, 1992; Ennen et al., 2010b). All measurements were divided by midline carapace length to scale for body size differences in our sample (Lovich and McCoy, 1992). All ratios were then arcsine-square-root transformed prior to analysis. Males and females were analyzed separately due to significant sexual size dimorphism in the genus (Gibbons and Lovich, 1990). Specimens that did not exhibit secondary sexual characters (i.e., smaller than the smallest male) were not used in these analyses.

Because of our small sample size of pure *G. barbouri* males from Ichawaynochaway Creek, we included two additional specimens that we identified as *G. barbouri* from the Choctawhatchee River and one from the Chattahoochee River in our discriminant analysis. For consistency, we included five specimens of diagnosable *G. barbouri* females from the Choctawhatchee drainage (Pea River) in discriminant analysis for that sex. Discrimination of groups is therefore expected to be conservative.

Genetics.—Total genomic DNA was extracted from the tissue samples with a DNeasy Tissue Kit (QIAGEN Inc., Valencia, CA). DNA was then stored at −20°C until use. We aligned published mitochondrial control region sequences for *G. ernsti* and *G. barbouri* (GenBank accession numbers GQ856218–GQ856220) using Sequencher 4.10.1 (GeneCodes Co., Ann Arbor, MI). From these sequences, we were able to identify a putatively diagnostic base substitution that could be used in a restriction fragment length polymorphism (RFLP) assay. The variable

Table 2. Names, abbreviated names, or acronyms, and presence/absence for qualitative head color characters used to discriminate between *Graptemys barbouri* and *Graptemys ernsti*.

Character	Abbreviated name/acronym	Species	
		Graptemys barbouri	*Graptemys ernsti*
Postorbital-interorbital connection	POB-IOB	present	absent
Supra-occipital blotches	SUPOC	absent	present
Subocular blotches	SUBOC	absent	present
Nasal trident		absent	present
Nasal arrow		present	absent
Middorsal heart-shaped blotch	Heart	present	absent
Transverse chin bar	Chin bar	present	absent
Lateral chin spots		absent	present

portion of the control region was then amplified using the primers described by Spinks and Shaffer (2005). Polymerase chain reactions (PCR) were performed with an Eppendorf Mastercyler in 25 μL reactions consisting of 1X *Taq* reaction buffer (New England Biolabs, Beverly, MA), 200 μM dNTPs, 2 mM $MgCl_2$, 0.5 units of *Taq* polymerase (New England Biolabs), 0.3 μM of each primer, approximately 20–100 ng template DNA, and water to the final volume. PCR cycling conditions consisted of an initial 1 min denaturing step at 95°C followed by 30 cycles of 1 min at 95°C, 1 min at 50°C, and 1 min at 72°C. A final elongation step of 7 min at 72°C completed the cycle. Restriction digests using *Dpn*II (New England Biolabs) were conducted following the manufacturer's recommendations in a 20 μL volume with 10 μL of the PCR product and were incubated at 37°C for 4 hrs. The restriction digests, along with a 100 bp size standard (New England Biolabs), were then visualized on 1.5% agarose gels stained with ethidium bromide (0.5 μg/ml). Individuals were then scored as having a haplotype of either *G. ernsti* or *G. barbouri*.

Selman et al. (2009) reported that several microsatellite loci originally developed for other emydid turtles were useful in three other species of *Graptemys*. We tested these loci in five individuals each of *G. ernsti* and *G. barbouri*. Six loci (*TerpSH1, TerpSH2*, *TerpSH5*, *GmuB08*, *GmuD51*, and *GmuD70*) produced reliable amplifications and were polymorphic. Polymerase chain reactions were performed on an Eppendorf Mastercyler in 12.5 μL reactions consisting of 1X *Taq* reaction buffer (New England Biolabs), 2 mM $MgCl_2$, 200 μM dNTPs, 0.1875 units of *Taq* polymerase (New England Biolabs), 0.16 μM of the M13 tailed forward primer (Schuelke, 2000), 0.16 μM of the reverse primer, 0.08 μM of the M13 labeled primer (LI-COR Inc., Lincoln, NE), 20–100 ng of template DNA, and water to the final volume. PCR cycling conditions consisted of an initial denaturing step of 94°C for 2 min followed by 35 cycles of 30 sec at 94°C, 1 min at 56°C, and 1 min at 72°C. A final elongation step of 10 min at 72°C ended the cycle. Microsatellite alleles were visualized using a LI-COR 4300 DNA sequencer and scored using a 50–350 bp size standard (LI-COR) and Gene Image IR v. 3.55 (LI-COR).

Genetic diversity measures for each site including the number of alleles (N_A), allelic richness (A_R), observed heterozygosity (H_O), expected heterozygosity (H_E), and the inbreeding coefficient (F_{IS}) were calculated by FSTAT 2.9.3 (Goudet, 1995). GENEPOP on the web v. 4.1 (Raymond and Rousset, 1995; Rousset, 2008) was used to test for Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium. A sequential Bonferroni correction (Rice, 1989) was used to adjust the *P*-values for multiple comparisons. Differences among sites were examined with a principal coordinates analysis (PCoA) of genetic distances among individuals as implemented by GenAlEx v. 6.5 (Peakall and Smouse, 2006, 2012).

The Bayesian approach employed by STRUCTURE 2.3.3 (Pritchard et al., 2000) was used to determine the number of genetically discrete populations (K) and to estimate the proportion of an individual's ancestry that originates from them. This allowed us to assess the distinctiveness of our collections of *G. ernsti* (Conecuh and Yellow rivers) and *G. barbouri* (Ichawaynochaway Creek) and to determine the ancestry of individuals in the Pea and Choctawhatchee rivers. We tested values of *K* from 1–6 without prior geographical information and assuming correlated allele frequencies with admixture between groups. For each value of *K,* we performed 20 independent runs with a burn-in of 100,000 followed by a subsequent 500,000 MCMC replications. The best value of *K* was determined by examining the probability scores for each value of *K* and by the Δ*K* method (Evanno et al., 2005) as calculated by Structure Harvester v 6.92 (Earl and von Holdt, 2011). We averaged all 20 runs at the best values of *K* with CLUMPP v. 1.1.2 (Jakobsson and Rosenberg, 2007), and visualized the results with DISTRUCT v. 1.1 (Rosenberg, 2004). The mean membership coefficient (q) was then used to determine whether an individual represented either of the two species or possessed mixed ancestry. Thresholds of the q scores to make these assignments were determined by simulation analysis (described below). We performed a separate STRUCTURE analysis of individuals of *G. ernsti* in order to determine if *G. ernsti* in the Pea River have been there long term or represent a recent introduction. The STRUCTURE analysis was performed as previously described except that we tested values of K ranging from 1–5. All *G. ernsti* from the Conecuh and Yellow rivers were included in the analysis along with eight individuals from the Pea River that were identified as *G. ernsti* (q scores > 0.92; choice of this threshold is described below) in the original STRUCTURE analysis.

Another Bayesian approach, as implemented by NewHybrids v. 1.0 (Anderson and Thompson, 2002), was used to examine the ancestry of individuals from the Pea and Choctawhatchee rivers. This program calculates the posterior probability that each individual belongs to one of six classes of genotypes including either one of the parental species or a hybrid (F1, F2, or a backcross between the F1 and either one of the parental species). Analyses were performed with either uniform or Jeffrey's priors for both allele frequencies and mixing proportion. Likewise, we ran the analysis both with and without *a priori* specification of individuals representing the parental species (i.e., the samples from rivers other than the Pea and Choctawhatchee). Each run included 100,000 sweeps for burn-in and 1,000,000 post burn-in iterations.

Simulations were employed to test the power of these two analyses to distinguish among individuals of pure and various hybrid ancestries given our data. Individuals of *G. ernsti* (Conecuh and Yellow rivers) and *G. barbouri* (Ichawaynochaway Creek) were considered to represent pure parental genotypes and establish allele frequencies in either species. We then used HybridLab v. 1.0 (Nielsen et al., 2006) to simulate 100 individuals representing each parent species and each type of hybrid. These simulated individuals were then analyzed in STRUCTURE and NewHybrids as described above. These results were used to establish thresholds for defining parental species in STRUCTURE and to assess the ability of NewHybrids to detect and classify individuals with hybrid ancestry.

RESULTS

Morphometrics.—POB-IOB connections are rarely present in *G. ernsti*. Only four of the specimens we examined from the Escambia-Yellow system ($n = 43$) possessed either a single or fully connected POB-IOB, and 88% individuals we examined lacked the connection on both sides. In contrast, *G. barbouri* from the Apalachicola drainage ($n = 29$) often possessed a fully connected POB-IOB (83%; Fig. 2D). In the Choctawhatchee drainage ($n = 34$), 76% lacked a connection, but 24% possessed full POB-IOB connections. Disregarding specimens with a single connection, the frequency of this variable in *Graptemys* from the Choctawhatchee differed significantly from *G. barbouri* ($\chi^2 = 23.22$, df = 1, $P < 0.0001$) but not *G. ernsti* ($\chi^2 = 1.82$, df = 1, $P = 0.18$).

Fig. 2. Diagnostic head characters of select *Graptemys* from the southeastern United States: (A) chin bar of *Graptemys barbouri* (Choctawhatchee River, Holmes County, FL), (B) chin spots of *Graptemys ernsti* (Yellow River, Covington County, AL), (C) POB-IOB separation, supraoccipital spots, and nasal trident of *G. ernsti* (Pea River, Geneva County, AL), (D) postorbital-interorbital (POB-IOB) connection, mid-dorsal "heart" pattern, and prefrontal arrow of *G. barbouri* (Choctawhatchee River, Geneva County, AL), and (E) intermediate dorsal head pattern of a *Graptemys* collected in the Choctawhatchee River drainage (Pea River, Geneva County, AL).

Graptemys barbouri typically possesses a transverse or curved bar of yellow pigmentation under the chin (Fig. 2A), and 97% of the specimens we examined ($n = 36$) possessed this character. In contrast, *G. ernsti* typically lacks a chin bar (Fig. 2B), and 81% of *G. ernsti* examined ($n = 48$) did not possess this character. *Graptemys* from the Choctawhatchee River drainage ($n = 34$) were intermediate: 59% possessed a chinbar, while 41% did not. Disregarding specimens with a partial bar, the frequency of this variable in *Graptemys* from the Choctawhatchee differed significantly from *G. barbouri* ($\chi^2 = 13.12$, df = 1, $P = 0.0003$) and *G. ernsti* ($\chi^2 = 16.15$, df = 1, $P < 0.0001$). The presence of a nasal trident is a diagnostic feature of *G. ernsti* (Fig. 2C); indeed, this pattern was present on 92% of specimens examined ($n = 59$). In contrast, none of the *G. barbouri* we examined possessed this pattern, which instead possessed a distinct prefrontal arrow (Fig. 2D). Again, specimens from the Choctawhatchee River drainage ($n = 35$) were intermediate with 29% in possession of and 71% lacking a nasal trident. Some of the specimens had odd tridents that looked like a prefrontal arrow (typical of *G. barbouri*) with disconnected prongs on the side (Fig. 2E), while others lacked the middle prong of the trident. The frequency of individuals possessing the nasal trident in *Graptemys* from the Choctawhatchee differed significantly from *G. barbouri* ($\chi^2 = 8.05$, df = 1, $P = 0.0045$) and *G. ernsti* ($\chi^2 = 37.22$, df = 1, $P < 0.0001$).

Supra-occipital blotches (SUPOC) are a diagnostic characteristic of *G. ernsti*, and 98% of individuals we examined from the Escambia-Yellow rivers ($n = 44$) exhibited this character. Conversely, only 3% of individuals examined from the Apalachicola drainage ($n = 35$) possessed SUPOC blotches. Individuals from the Choctawhatchee drainage ($n = 26$) possessed both character states: 58% possessed SUPOC blotches, while 42% did not. The frequency of individuals possessing SUPOC blotches in *Graptemys* from the Choctawhatchee differed significantly from *G. barbouri* ($\chi^2 = 20.44$, df = 1, $P < 0.0001$) and *G. ernsti* ($\chi^2 = 15.73$, df = 1, $P < 0.0001$).

Subocular blotches (SUBOC) are present with greater frequency in *G. ernsti* that *G. barbouri*: none of the *G. barbouri* examined ($n = 35$) possessed SUBOC blotches, whereas 29% of the *G. ernsti* examined ($n = 45$) possessed this character. Only 7% of individuals examined from the Choctawhatchee drainage ($n = 27$) exhibited SUBOC blotches. The frequency of individuals possessing SUBOC spots in *Graptemys* from the Choctawhatchee did not differ from *G. barbouri* ($\chi^2 = 4.09$, df = 2, $P = 0.130$) or *G. ernsti* ($\chi^2 = 4.87$, df = 2, $P = 0.088$).

A final diagnostic feature of *G. barbouri* is the presence of a heart-shaped pattern on the top of the head posterior to the orbits. Of individuals we examined ($n = 15$), 80% possessed the heart. Conversely, none of the *G. ernsti* we examined ($n = 25$) possessed the heart-shaped mark, while 56% of the *Graptemys* from the Choctawhatchee drainage did ($n = 36$). The frequency of individuals possessing the heart-shaped pattern in *Graptemys* from the Choctawhatchee differed significantly from *G. ernsti* ($\chi^2 = 20.52$, df = 1, $P < 0.0001$) but not *G. barbouri* ($\chi^2 = 0.98$, df = 1, $P = 0.32$).

A discriminant function using the eight mensural shell variables correctly classified 24 out of 26 male specimens. The function was statistically significant at discriminating among the taxa (Wilks' Lambda = 0.17, $F = 2.83$, df = 16, 32, $P = 0.006$). Male *G. barbouri* and *G. ernsti* show distinct clusters when plotting discriminant scores, with *Graptemys* from the Choctawhatchee River overlapping both species on the first discriminant axis and especially *G. ernsti* on the second discriminant axis (Fig. 3A). A similar function correctly classified 49 out of 64 female specimens. That function was also statistically significant (Wilks' Lambda = 0.34, $F = 4.89$, df = 16, 108, $P < 0.001$). Female *G. barbouri*

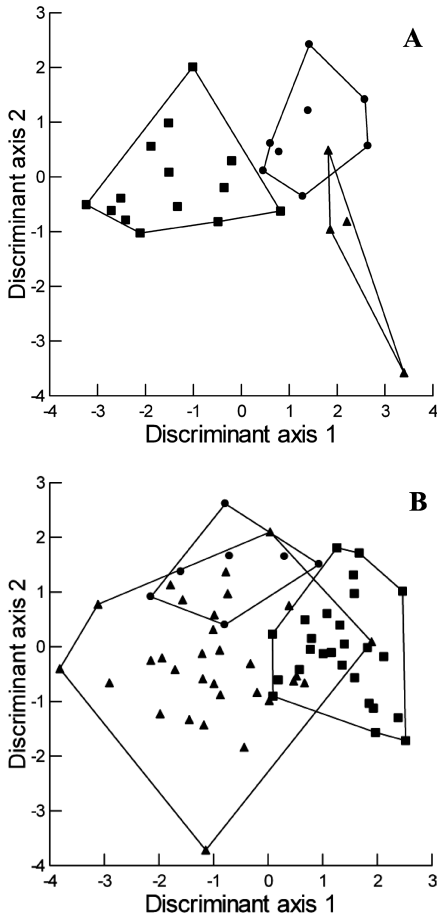

Fig. 3. Plot of discriminant scores for mensural shell variables in male (A) and female (B) *Graptemys*. Refer to text for details. Symbols are as follows: squares = *Graptemys ernsti*, triangles = *Graptemys barbouri*, and solid circles = *Graptemys* from the Choctawhatchee and Pea rivers. Minimum convex polygons are drawn around each cluster of points.

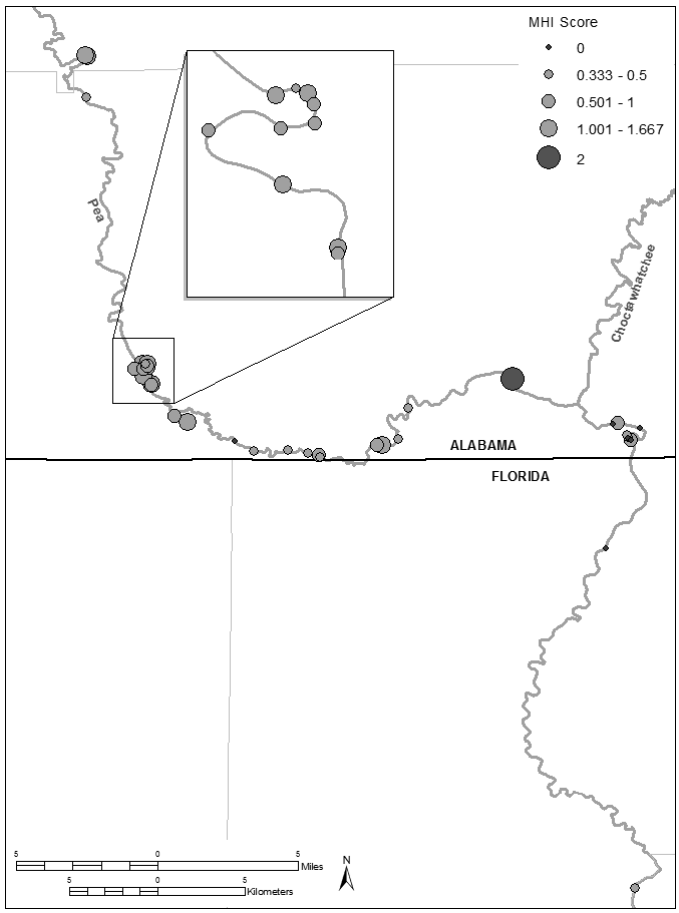

Fig. 4. Distribution of MHI scores of individuals from the Choctawhatchee and Pea rivers, Alabama and Florida, ranging from 2 (morphologically pure *Graptemys ernsti*) to 0 (morphologically pure *Graptemys barbouri*).

and *G. ernsti* show less separation than males, but still discernible clusters, when plotting discriminant scores. *Graptemys* from the Choctawhatchee River overlap parental species to some degree on both discriminant axes (Fig. 3B).

The average MHI scores varied among drainages (Fig. 4; Table 3). As expected, the individuals from the Escambia-Yellow system had high average MHI scores of 1.63 (0.23) and 1.57 (0.33), respectively. Likewise, individuals from the Ichawaynochaway Creek population had a low average MHI score of 0.04 (0.13). The individuals from the Choctawhatchee River had an average MHI of 0.22 (0.31), which suggests these individuals are more *barbouri*-like in their morphology. For example, eight out of 15 individuals in the Choctawhatchee River scored an average MHI of 0.0, a morphologically pure *G. barbouri*, while five out of 15 scored an average MHI between 0.74 and 0.25, representing predominantly characteristics of *G. barbouri*. Only two individuals from the Choctawhatchee River were scored as intermediate (i.e., MHI of 1.24–0.75). Interestingly, the average MHI score of individuals from the Pea River was 0.87 (0.52), which suggests morphologies here are mostly an intermediate form. About half ($n = 12$) of the individuals in the Pea River had MHI scores that were between 0.74–0.25 or predominantly characteristics of *G. barbouri*. Only one individual in the Pea River had a MHI of 0.0 as a morphologically pure *G. barbouri*. Six out of 29 individuals from the Pea River were morphologically more similar to *G. ernsti* than *G. barbouri* (MHI scores: 1.75–1.25), and one individual was morphologically pure *G. ernsti*. The Pea River had nine individuals with MHI scores between 1.24 and 0.75 and considered possessing intermediate morphologies. MHI scores differed significantly among the five drainage systems ($K = 78.05$, df = 4, $P < 0.001$; Table 3), and a pairwise comparison found MHI scores to be significantly higher in the Pea than in the Choctawhatchee ($W = 15.12$, df = 1, $P < 0.001$).

Genetics.—PCR amplification of the control region produced a 707 bp fragment. A total of 132 individuals (Appendix 1) were screened for the putatively diagnostic restriction site differences that defined the haplotypes found in the two species. The robustness of our mtDNA marker was supported by the observation that all individuals from the Conecuh/Yellow and Ichawaynochaway had restriction digestion profiles of the predicted size for *G. ernsti* (419 and 288 bp) and *G. barbouri* (288, 238, and 181 bp), respectively. No intraspecific variation in the digest pattern for this restriction enzyme was detected in either species. Collections from the Choctawhatchee and Pea rivers contained both haplotypes. Within the Choctawhatchee, the *G. barbouri* haplotype was most common (10 of 13 individuals), while the *G. ernsti* haplotype had the highest frequency in the Pea River (20 of 29 individuals).

Each microsatellite locus had alleles that were exclusive to one or the other of the two species, and two loci (*TerpSH2* and *GmuD51*) possessed alleles that were diagnostic for

Table 3. A comparison between the average morphological hybrid index (MHI) and the average q score by river. The MHI scores are on a 0–2 scale, where a 0 represents *Graptemys barbouri* and a 2 represents *Graptemys ernsti*. Numbers in parentheses are the standard deviations.

River	MHI	q score
Ichuawaynochaway	0.04 (0.10)	0.009 (0.005)
Conecuh	1.65 (0.25)	0.99 (0.01)
Yellow	1.58 (0.38)	0.99 (0.001)
Choctawhatchee	0.17 (0.34)	0.03 (0.04)
Pea	0.90 (0.50)	0.49 (0.41)

either *G. ernsti* or *G. barbouri* (Appendix 2). After sequential Bonferroni correction, only *TerpSH1* was found to deviate from HWE in the Ichawaynochaway Creek and Pea River sites. Similarly, after correction, only one case of linkage disequilibrium was found (Pea River; *TerpSH2* and *GmuB08*). Genetic diversity measures averaged across loci were fairly consistent across all sites except for the Yellow River (Table 4), which had a substantially smaller average number of alleles and lower heterozygosity values. Average F_{IS} values were close to zero for all sites other than the Pea River (Table 4). The average F_{IS} of 0.203 for the Pea River reflects a deficit in the observed number of heterozygotes compared to the expected. The PCoA (Fig. 5) revealed two groups comprised of individuals representing *G. ernsti* from the Conecuh and Yellow rivers and *G. barbouri* from the Ichawaynochaway Creek. Most individuals from the Pea and Choctawhatchee rivers fell within one of these two groups, although a few were located across the middle of the ordination.

The Bayesian analysis performed by STRUCTURE detected two strongly supported genetic groups representing *G. barbouri* and *G. ernsti*. At $K = 2$, the likelihood scores reached an asymptote (average lnL = −2291.75; SD = 0.18), also corresponding to the peak in the ΔK scores. Individuals from the Conecuh and Yellow rivers (*G. ernsti*) had an average membership score (q) for group one of 0.991 (±0.002) with a range of 0.944–0.995. In Ichawaynochaway Creek, the average membership score for group two (*G. barbouri*) was also 0.991 (±0.002) with a range of 0.973–0.994. The membership scores for each individual (Appendix 1) were greater than the average values for *G. ernsti* (0.924±0.004) or *G. barbouri* (0.917±0.007) found in the simulated data (Table 5). Within the Choctawhatchee River, 11 of the 13 individuals (Appendix 1; Fig. 6) possessed q scores for the *G. barbouri* group of >0.92. For the Pea River (Appendix 1; Fig. 6), nine individuals would classify as *G. barbouri* and seven would classify as *G. ernsti* based on the q threshold of 0.92, while the 13 remaining individuals showing mixed ancestry (q < 0.92 for either group). However, the simulation analysis revealed that STRUCTURE had little power to infer the extent of mixed ancestry in an individual. The average q score for the F1 hybrids was 0.498 (±0.12) with a range of 0.372–0.645 (Table 5). Backcrosses became even more problematic as the range of q scores in the simulated data included values that would qualify as a pure species in the simulated data (q > 0.92). In the STRUCTURE analysis of only individuals of *G. ernsti*, the likelihood scores plateaued at K = 3 (average lnL = −887.33; SD = 1.04), where each site seemed to represent its own genetically distinct group. Within each site, the average q scores for each respective group were 0.878 (±0.006) in the Conecuh, 0.947 (±0.026) in the Yellow, and 0.830 (±0.026) in the Pea.

The NewHybrids analysis of the simulated data demonstrated that there was a high probability of identifying parental species and F1 individuals (average probability > 0.99; Table 5). However, there was considerably less power to accurately classify F2 hybrids and backcrosses. In the simulated data, even when using a more permissive probability value to assign an individual to a category (>0.9), only 58%, 34%, and 67% of the individuals were correctly classified as F2s, *G. ernsti*-backcross, and *G. barbouri*-backcross, respectively (data not shown). Using a probability threshold of >0.99, seven individuals in the Choctawhatchee River classified as *G. barbouri*, although the remaining individuals all also had the highest probability in this category (Appendix 1; Fig. 5). In the Pea River, ten individuals had a high probability of being one of the parental species (six = *G. barbouri*; four = *G. ernsti*), while seven and six individuals, respectively, had their highest probability in these two categories. No individual had a higher probability than 0.14 of being an F1 hybrid. The remaining individuals had their highest probability as being an F2 hybrid ($n = 1$), *G. barbouri*-backcross ($n = 3$), or *G. ernsti*-backcross ($n = 2$; Appendix 1; Fig. 5). Also, q scores and MHI were positively correlated ($r = 0.92$, $df = 95$, $t = 22.90$, $P < 0.001$), suggesting that mixed ancestry individuals based on genetic data also had intermediate morphologies.

DISCUSSION

Both our molecular and morphological analyses identified pure individuals of *Graptemys ernsti* and *Graptemys barbouri* along with individuals of varying degrees of introgression within the Choctawhatchee and Pea rivers. Our study presents the first example of two syntopic megacephalic *Graptemys*, an exception to the well-documented pattern of drainage-specific endemism in this genus, and the first to report currently active natural hybridization within the

Table 4. The number of samples from each site (n) and their genetic diversity measures averaged across the six microsatellite loci used in this study. The sites represent the rivers where the samples were collected. N_A represents the number of alleles, A_R is the allelic richness, H_O is the observed heterozygosity, H_E is the expected heterozygosity, and F_{IS} is the inbreeding coefficient.

River	n	N_A	A_R	H_O	H_E	F_{IS}
Conecuh	33	5.67	4.47	0.621	0.595	−0.03
Yellow	27	3.5	3.06	0.4	0.396	0.011
Ichawaynochaway	30	7.33	5.81	0.702	0.731	0.062
Choctawhatchee	13	5.5	5.3	0.661	0.627	−0.013
Pea	29	6.67	5.96	0.617	0.759	0.203

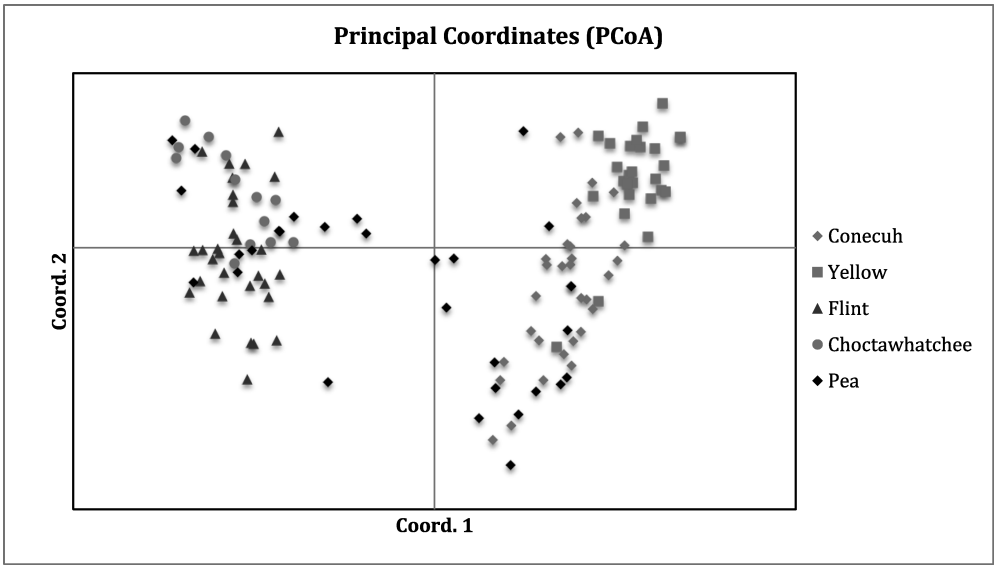

Fig. 5. Principal coordinate analysis showing allelic ordination of *Graptemys* from Ichawaynochaway Creek, Georgia and the Conecuh, Yellow, Pea, and Choctawhatchee rivers of Alabama and Florida.

pulchra clade. Examples of natural hybridization of terrestrial and freshwater turtle species are known, but reports of introgression are few (Crenshaw, 1965; Ward, 1968; Lutterschmidt et al., 2007). However, an historical (>200 years) hybridization and introgression event was detected between *Graptemys geographica* and *Graptemys pseudogeographica* in Tennessee (Freedberg and Myers, 2012), where introgression appears to have been directionally limited to the mixing of *G. geographica* haplotypes into *G. pseudogeographica*.

Hybridization is not the only process that can produce individuals showing mixed ancestry. Incomplete lineage sorting (ILS) can also lead to gene tree incongruence, and a variety of analytical techniques have been developed to distinguish between the two (e.g., Holland et al., 2008; Joly et al., 2009). Unfortunately, in our study, we lack the sequence data from numerous nuclear genes required to use these approaches. However, we have other reasons to suggest that our microsatellite data do indeed reflect hybridization between *G. ernsti* and *G. barbouri* rather than ILS. If there was ILS between *G. ernsti* and *G. barbouri*, then one might expect the microsatellite loci to demonstrate a relatively high frequency of shared alleles. In fact, this is far from the case, as across all loci only 15 alleles were shared between species compared to the 43 alleles that were present in only one species (Appendix 2). Overall, five of the six loci had more unique alleles than ones that were shared. In these comparisons, we have only considered sites representing the ''pure'' parental species.

We also note that the STRUCTURE analysis and the PCoA clearly distinguished the two species, both from allopatric and sympatric sites. The ancestry for most individuals within the Pea and almost all of individuals within the Choctawhatchee River was strongly assigned to one of the two species (Appendix 1). Similarly, only a few individuals (e.g., numbers 13, 17, and 24) fall within the middle of the ordination (Fig. 5), reflecting individuals of mixed ancestry. If ILS was at work within this drainage then one might expect to see more individuals with evidence of mixed ancestry and for these individuals to be present in both parts of the drainage.

Geographic variation in capture frequency in our study is suggestive of a non-random distribution of each species and

Table 5. Results of the STRUCTURE and NewHybrids analyses of the simulated data sets. For the STRUCTURE analysis, the average q score for the *G. ernsti* group is reported for all of the categories except the simulated *G. barbouri* and *G. barbouri* BC classes. For the NewHybrids analysis, the probability of belonging to a particular genotype class is reported. Only the results of the analaysis that did not start with prior information on parental species are reported. The 95% confidence intervals (95% CI) and minimum (MIN) and maximum (MAX) values are also reported for both sets of analyses.

Software/Statistic	*G. ernsti*	*G. barbouri*	F1	F2	*G. ernsti* BC	*G. barbouri* BC
STRUCTURE						
Average q score	0.924	0.917	0.498	0.506	0.728	0.707
95% CI	0.004	0.007	0.012	0.031	0.021	0.023
MIN	0.829	0.785	0.372	0.123	0.452	0.344
MAX	0.946	0.946	0.645	0.877	0.935	0.946
NewHybrids						
Average probability	0.999	0.998	0.996	0.827	0.699	0.821
95% CI	0.0002	0.0009	0.001	0.052	0.054	0.051
MIN	0.9917	0.9666	0.9305	0.021	0.0322	0.0178
MAX	0.9997	0.9998	0.9992	0.9994	0.9815	0.9915

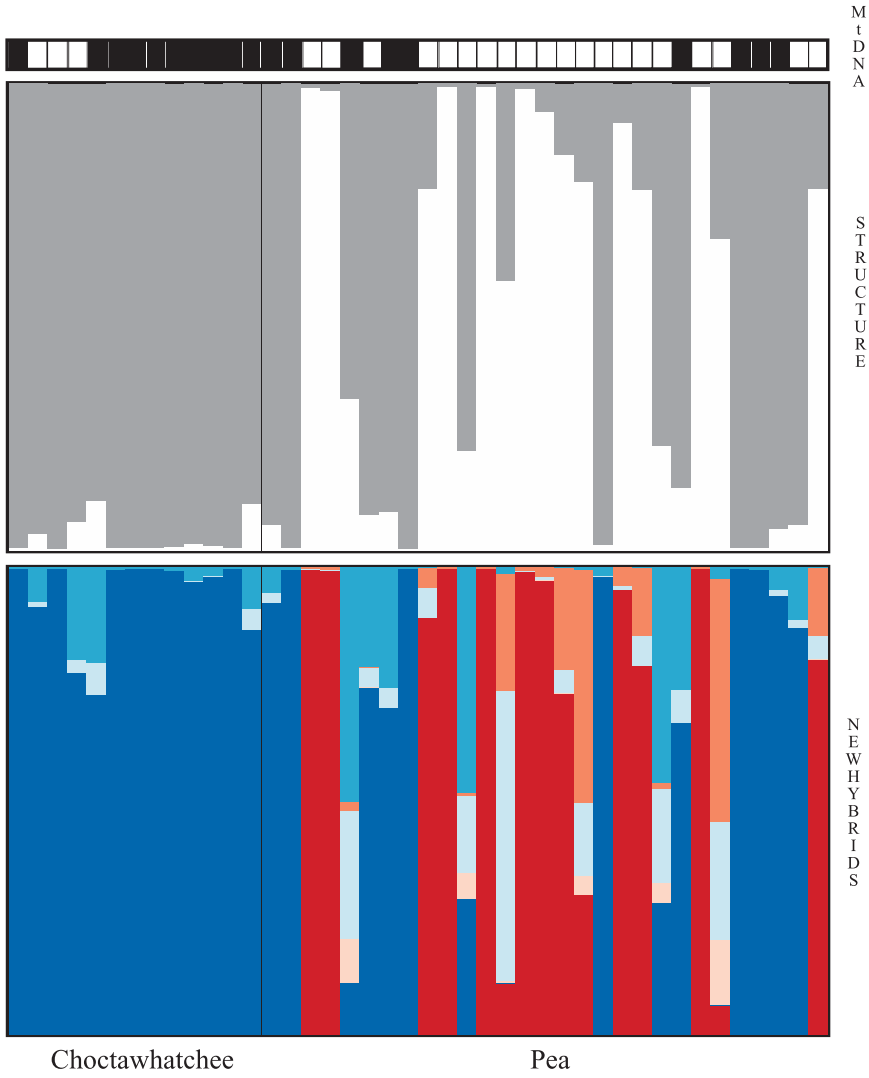

Fig. 6. Bar plots showing the results of the mtDNA assay, STRUCTURE analysis, and NewHybrids analysis for 42 *Graptemys* from the Choctawhatchee and Pea rivers, Alabama and Florida. The order of individuals corresponds to that in Appendix 1. For the mtDNA, black represents *G. barbouri* and white represents *G. ernsti* haplotypes. The STRUCTURE results show the average ancestry scores (q) with gray representing the *G. barbouri* group and white the *G. ernsti* group. The NewHybrids plot shows the probability of an individual belonging to one of the six genotype classes with colors ranging from dark blue to dark red reflecting *G. barbouri*, *G. barbouri*-backcross, F2, F1, *G. ernsti*-backcross, and *G. ernsti*, respectively.

hybrids throughout the Choctawhatchee drainage. The *G. barbouri* mtDNA haplotype and morphology (i.e., average MHI score of 0.17) were most prevalent in specimens collected below the confluence of the Choctawhatchee and Pea rivers. Conversely, specimens from the Pea River, upstream from the confluence of the two rivers, were represented by individuals with a much higher degree of *G. ernsti* ancestry (Fig. 4). Some individuals possessed intermediate morphologies (i.e., average MHI score of 0.90) and q scores, while others appeared to represent relatively pure *G. ernsti* (Table 3). Thus, genetic and morphological evidence suggests that *G. ernsti* and hybrid individuals are largely limited to the Pea River. However, additionally distributional work is needed to determine the abundance of *G. barbouri*, *G. ernsti*, and hybrids throughout the entire Choctawhatchee River drainage.

The non-random distribution of *G. barbouri*, *G. ernsti*, and hybrids within the Choctawhatchee and Pea rivers could be explained by interspecific competition. Female megacephalic *Graptemys* are strongly molluscivorous (Lindeman, 2000), a prey resource that has experienced significant (50%) declines in the Choctawhatchee and Pea rivers (Williams et al., 2008). Because these two species have the same trophic niche and molluscan abundances appear relatively low, competitive interactions over potentially limited food resources may also occur. However, interpretations of co-occurrence patterns to infer competition are tenuous (Hastings, 1987), and non-random distributions can be influenced by stochastic drift processes (Ulrich, 2004). To generate evidence that competition may structure turtle assemblages in the Choctawhatchee River drainage or elsewhere, we recommend that workers use multiple approaches, including appropriate null models to demonstrate non-random distribution (Gotelli and Graves, 1996) and other independent evidence of interspecific interactions, such as resource partitioning (e.g., Gotelli and Ellison, 2002).

The origin of *Graptemys* in the Choctawhatchee River drainage is open to question. No *Graptemys* were reported from the Choctawhatchee River drainage during the last century, despite extensive turtle collections throughout the southeastern United States (summarized in Ernst and

Lovich, 2009; Mount, unpubl. data). A history of research on the genus *Graptemys* was provided by Lindeman (2013). Early researchers including Cagle, Tinkle, Vogt, McCoy, Mount, and Dobie seemed to bypass or ignore the Choctawhatchee River system based on available field notes and recollections, some on the assumption that *Graptemys* were not there. Even though two specimens of *G. barbouri* were collected on the Choctawhatchee at the Hwy. 90 crossing in Florida on 5 July 1965 and catalogued into the Auburn University collection (AUM 3880–3881), Mount disregarded those specimens in his book on Alabama amphibians and reptiles (Mount, 1975) for unknown reasons. According to Lindeman (pers. comm.), much of the earlier turtle research in the area was focused below rather than above the Florida-Alabama line (until Shealy, 1976). In addition, there are few good access points on the Choctawhatchee, and *Graptemys* are not as plentiful or easily observed there as they are on other rivers in the region. We believe these factors contributed to the fact that *Graptemys* were essentially overlooked in the Choctawhatchee River for so long.

The null hypothesis for the presence of *Graptemys* in the Choctawhatchee River drainage is that they were historically present but overlooked due to limited collecting effort. Collection methods in the last century were by foot along river banks, canoeing, or swimming rivers, with canoeing being the only technique to lend itself well to the collection of *Graptemys*. Individuals, especially females, are extremely wary and easily disturbed while basking (JCG, pers. obs.). Further, encounter rates of basking *Graptemys* during canoe surveys (individuals/river km) suggest that *Graptemys* are less abundant in the Choctawhatchee drainage than other drainages in Alabama (Godwin, unpubl. data) and in the Choctawhatchee drainage in Florida (Enge and Wallace, 2008). Thus, perhaps early collection efforts were too sparse to detect *Graptemys* in this drainage, where turtles occur in relatively low densities.

An alternative hypothesis to explain the syntopic occurrence of megacephalic *Graptemys* in the Choctawhatchee drainage involves human-mediated introduction. This could entail either one species being naturally present in the system with the other being introduced, or both species being introduced. However, this explanation is not supported by the skewed spatial distribution of *G. barbouri*, *G. ernsti*, and hybrids within the drainage. Generation time for *G. ernsti* is 14 years (Shealy, 1976). The time from the late 1960s to the late 1990s would allow for two generations, an unlikely time period to allow for turtles to infiltrate dozens of stream kilometers of habitable range and produce a viable population without numerous individuals being anthropogenically introduced. Bridge crossings, the most likely points for introduction, on both rivers are few and separated by wide distances. Additionally, analysis of the microsatellite data (not shown) of the populations of *G. ernsti* (i.e., Yellow, Conecuh, and Pea rivers) indicate that each is unique; thus, the Pea River population is not due to a recent invasion or human-mediated introduction from either the Conecuh or Yellow rivers. While the syntopy of *G. barbouri* and *G. ernsti* counters the general pattern of drainage-specific endemism, microsatellite divergence of the populations of *G. ernsti* tends to support the pattern.

The origin and speciation of *Graptemys* is poorly understood because fossil evidence is scant (Wilson and Zug, 1966; Jackson, 1975; Holman et al., 1990; Ehret and Bourque, 2011). Currently no fossil records of *Graptemys* are known from the Choctawhatchee River drainage. Further, while the current state of testudine mitochondrial DNA and nuclear DNA analyses are able to resolve broad-scale phylogenies, resolution of intrageneric relationships have been challenging (Spinks and Shaffer, 2009; Barley et al., 2010; Wiens et al., 2010; Spinks et al., 2013). Available fossil evidence indicates that speciation of *Graptemys* may have peaked during the Pleistocene. *Graptemys pseudogeographica* occurred as far north as central Michigan (Wilson and Zug, 1966), *Graptemys geographica* has undergone post-Pleistocene range reduction in Kansas and extirpation from Texas (Holman, 1980; Ernst and Lovich, 2009; Collins et al., 2010), and the Waccasassa and Suwannee rivers were occupied by the megacephalic *Graptemys kerneri* (Fig. 1; Jackson, 1975; Ehret and Bourque, 2011).

Evolution and diversification in the genus *Graptemys* is putatively linked to sea level fluctuations in the southeastern United States. These fluctuations occurred during glacial and interglacial periods starting in the early or middle Miocene extending through the Pleistocene as reviewed by Lamb et al. (1994). Sea level fluctuations caused periodic isolation and integration of various river systems along the Gulf Coast, and this may have facilitated the movement of *G. ernsti* and *G. barbouri* into the Choctawhatchee River drainage. There is evidence that barrier islands existed well offshore of the present strand position in Choctawhatchee Bay during the Late Wisconsin regression (Hyne and Goodell, 1967); therefore, river channels and mouths would have extended beyond their current limits, and *G. barbouri* may have entered the Choctawhatchee River through what is now Choctawhatchee Bay when it was not inundated by sea water. Jackson (1975) presents a second mechanism to explain distribution of *Graptemys*; flood events in upper stream reaches may have allowed *G. barbouri* to cross drainages. Also, the combination of these events could have facilitated dispersal of *G. barbouri* from the Chattahoochee or Chipola rivers into the Choctawhatchee River.

Evidence of paleo-river systems was provided by Locker and Doyle (1992) for the area. They identified four Plio-Pleistocene fluvial-deltaic systems that provided sediment inputs to the northwest Florida inner continental shelf. These included the Santa Rosa Island system between Pensacola Bay (the terminus for the Escambia-Yellow system where *G. ernsti* occurs) and Choctawhatchee Bay. They attributed deltaic deposits to either a paleo-Escambia-Yellow system or a paleo-Choctawhatchee River system. However, Locker and Doyle (1992) also suggested the possibility that both were part of a large system related to the present Choctawhatchee River. If this were the case, it may have allowed *G. ernsti* to invade the Choctawhatchee River during the Pliocene or the Pleistocene.

A final hypothesis for the presence of *G. ernsti* in the Pea River, given the current pattern of drainages, is a stream-capture event. The major downstream drainage pattern of the Pea, Yellow, and Conecuh rivers is a trend to the southwest. Streams of these drainages exhibit asymmetrical terrace development of extensive terraces on the northwest and steep slopes with little to no terracing on the southeast. During the Pliocene-Pleistocene period the Pea and Conecuh rivers may have migrated to their present positions (Price and Whetstone, 1977). Lightwood Knot Creek is the major eastern headwater tributary of the Yellow River that also drains from northeast to southwest and is presently separated from the Pea River by a linear distance of approximately 10 km. The general channel orientation

suggests that historically the upper reaches of the Pea River were once connected to Lightwood Knot Creek. During the Pliocene-Pleistocene southerly migration of rivers, the upper Pea River may have become disconnected from the Yellow River and joined the Choctawhatchee River, thus bringing *G. ernsti* into contact with *G. barbouri*.

Although the interaction between sea level fluctuations and paleo-river locations may have facilitated both movement and isolation of various species of *Graptemys* through the Plio-Pleistocene, it may be puzzling that despite our evidence of backcross ancestry we did not detect any F1 hybrids. However, this phenomenon is not unusual, as other studies reported similar findings for mammals (Red and Sitka deer: Goodman et al., 1999; Eastern and Western Grey Kangaroo: Neaves et al., 2010). Goodman et al. (1999) suggested, ''one expects twice as many first-generation backcrosses, four times as many second-generation backcross, and so on,'' which could explain our lack of F1 hybrids due to a small sample size or sampling multiple generations.

The primary isolating mechanism preventing hybridization in Gulf Coast species of *Graptemys* is allopatry. Courtship of *Graptemys barbouri* and *G. ernsti* have only been observed in captivity, each exhibiting similar behaviors (Wahlquist, 1970; Shealy, 1976). We believe that the initial phases of courtship of these two species are sufficiently similar that pre-zygotic isolating mechanisms could be compromised, resulting in interspecific mating, hybrid offspring, and introgression in the Choctawhatchee River drainage. Increased turbidity resulting from siltation and river degradation due to agriculture (Witmer et al., 2009) accompanied by reductions in underwater visibility may also be a factor in the breakdown of pre-zygotic isolating mechanisms (e.g., behavioral cues).

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Appendix 1. Individuals included in this study listed by site along with their mtDNA haplotype (E for *G. ernsti* and B for *G. barbouri*). The average q score from the STRUCTURE analysis is also reported for the groups representing *G. ernsti* (E) and *G. barbouri* (B). The probability of membership in one of the six genotype classes (E = *G. ernsti*, B = *G. barbouri*, F1 hybrid, F2 hybrid, E-BC = *G. ernsti*-backcross, and B-BC = *G. barbouri*-backcross), from the NewHybrids analysis is reported. MHI scores are also presented.

			STRUCTURE		NewHybrids						
Code	River	mtDNA	E	B	E	B	F1	F2	E-BC	B-BC	MHI
1	Conecuh	E	0.994[b]	0.006	0.998	0	0	0	0.002	0	1.6
2	Conecuh	E	0.988[b]	0.012	0.994	0	0	0	0.006	0	1.6
3	Conecuh	E	0.993[b]	0.007	0.996	0	0	0	0.004	0	2
43	Conecuh	E	0.944[b]	0.056	0.958	0	0	0.005	0.037	0	1.667
44	Conecuh	E	0.994[b]	0.006	0.998	0	0	0	0.002	0	2
45	Conecuh	E	0.991[b]	0.009	0.994	0	0	0.001	0.006	0	1.667
46	Conecuh	E	0.994[b]	0.006	0.998	0	0	0	0.002	0	1.667
48	Conecuh	E	0.944[b]	0.057	0.983	0	0	0.002	0.015	0	1.667
49	Conecuh	E	0.993[b]	0.007	0.997	0	0	0	0.003	0	1.667
77	Conecuh	E	0.991[b]	0.009	0.994	0	0	0.001	0.005	0	2
78	Conecuh	E	0.993[b]	0.007	0.997	0	0	0	0.003	0	2
79	Conecuh	E	0.991[b]	0.009	0.993	0	0	0.001	0.006	0	1.6
80	Conecuh	E	0.993[b]	0.007	0.996	0	0	0	0.003	0	1.2
81	Conecuh	E	0.994[b]	0.006	0.998	0	0	0	0.002	0	—
82	Conecuh	E	0.992[b]	0.009	0.995	0	0	0	0.005	0	—
83	Conecuh	E	0.994[b]	0.006	0.998	0	0	0	0.002	0	—
84	Conecuh	E	0.993[b]	0.007	0.997	0	0	0	0.003	0	—
85	Conecuh	E	0.994[b]	0.006	0.997	0	0	0	0.002	0	—
126	Conecuh	E	0.992[b]	0.008	0.996	0	0	0	0.004	0	1.667
127	Conecuh	E	0.992[b]	0.008	0.998	0	0	0	0.002	0	1.667
128	Conecuh	E	0.991[b]	0.009	0.994	0	0	0.001	0.006	0	1.667
129	Conecuh	E	0.99[b]	0.01	0.997	0	0	0	0.003	0	1.667
130	Conecuh	E	0.983[b]	0.017	0.991	0	0	0.001	0.008	0	1.333
131	Conecuh	E	0.993[b]	0.007	0.997	0	0	0	0.003	0	1.667
133	Conecuh	E	0.993[b]	0.007	0.997	0	0	0	0.003	0	1.667
134	Conecuh	E	0.993[b]	0.007	0.996	0	0	0	0.003	0	1.667
135	Conecuh	E	0.992[b]	0.008	0.996	0	0	0	0.004	0	1.667
136	Conecuh	E	0.99[b]	0.011	0.994	0	0	0.001	0.006	0	1.333
137	Conecuh	E	0.992[b]	0.008	0.995	0	0	0	0.004	0	1.333
138	Conecuh	E	0.991[b]	0.009	0.994	0	0	0.001	0.005	0	1.5
139	Conecuh	E	0.976[b]	0.024	0.995	0	0	0	0.004	0	1.667
140	Conecuh	E	0.991[b]	0.009	0.998	0	0	0	0.002	0	2
141	Conecuh	E	0.991[b]	0.009	0.993	0	0	0.001	0.006	0	1.333
47	Yellow	E	0.993[b]	0.007	0.997	0	0	0	0.003	0	2
50	Yellow	E	0.994[b]	0.006	0.998	0	0	0	0.002	0	1.333
51	Yellow	E	0.995[b]	0.005	0.999	0	0	0	0.001	0	1.667
52	Yellow	E	0.994[b]	0.006	0.998	0	0	0	0.002	0	—
53	Yellow	E	0.994[b]	0.006	0.998	0	0	0	0.002	0	2
54	Yellow	E	0.994[b]	0.007	0.997	0	0	0	0.003	0	—
55	Yellow	E	0.995[b]	0.005	0.999	0	0	0	0.001	0	—
56	Yellow	E	0.995[b]	0.005	0.999	0	0	0	0.001	0	1.667
57	Yellow	E	0.994[b]	0.006	0.998	0	0	0	0.002	0	2
58	Yellow	E	0.994[b]	0.006	0.998	0	0	0	0.002	0	1
59	Yellow	E	0.995[b]	0.005	0.999	0	0	0	0.001	0	—
60	Yellow	E	0.994[b]	0.006	0.998	0	0	0	0.002	0	1.2
61	Yellow	E	0.992[b]	0.008	0.996	0	0	0	0.004	0	—
62	Yellow	E	0.993[b]	0.007	0.997	0	0	0	0.003	0	1.2
64	Yellow	E	0.995[b]	0.005	0.999	0	0	0	0.001	0	2
65	Yellow	E	0.994[b]	0.006	0.998	0	0	0	0.002	0	—
66	Yellow	E	0.994[b]	0.006	0.998	0	0	0	0.002	0	—
67	Yellow	E	0.994[b]	0.006	0.998	0	0	0	0.002	0	—
68	Yellow	E	0.992[b]	0.008	0.996	0	0	0	0.004	0	—
69	Yellow	E	0.995[b]	0.005	0.999	0	0	0	0.001	0	1.6
70	Yellow	E	0.994[b]	0.006	0.998	0	0	0	0.002	0	—
71	Yellow	E	0.994[b]	0.006	0.998	0	0	0	0.002	0	1.6
72	Yellow	E	0.994[b]	0.006	0.998	0	0	0	0.002	0	—

Appendix 1. Continued.

			STRUCTURE		NewHybrids						
Code	River	mtDNA	E	B	E	B	F1	F2	E-BC	B-BC	MHI
73	Yellow	E	0.994[b]	0.006	0.998	0	0	0	0.002	0	—
74	Yellow	E	0.994[b]	0.006	0.998	0	0	0	0.002	0	—
75	Yellow	E	0.995[b]	0.005	0.999	0	0	0	0.001	0	1.6
76	Yellow	E	0.994[b]	0.006	0.998	0	0	0	0.002	0	—
4	Pea	B	0.056	0.944[b]	0	0.924	0	0.022	0	0.054	0.8
5	Pea	B	0.006	0.994[b]	0	0.996[b]	0	0	0	0.004	0.4
6	Pea	E	0.992[b]	0.008	0.996[b]	0	0	0	0.004	0	0.8
7	Pea	E	0.985[b]	0.015	0.993[b]	0	0	0.001	0.006	0	1.2
8	Pea	B	0.326	0.675	0	0.112	0.093	0.274	0.021	0.501	0.4
9	Pea	E[a]	0.078	0.922[b]	0	0.742	0.001	0.043	0	0.214	—
10	Pea	B	0.084	0.916	0	0.699	0.001	0.042	0	0.258	—
11	Pea	B	0.005	0.995[b]	0	0.997[b]	0	0	0	0.003	0.5
15	Pea	E	0.774	0.226	0.893	0	0	0.063	0.044	0.001	1.2
16	Pea	E	0.993[b]	0.007	0.997[b]	0	0	0	0.003	0	1.667
17	Pea	E	0.214	0.787	0	0.291	0.055	0.164	0.006	0.484	0.333
21	Pea	E	0.994[b]	0.006	0.998	0	0	0	0.002	0	1.667
22	Pea	E	0.577	0.423	0.11	0	0.005	0.622	0.249	0.014	1.333
23	Pea	E	0.989[b]	0.011	0.99[b]	0	0	0.001	0.008	0	1.667
24	Pea	E	0.939[b]	0.061	0.973	0	0	0.006	0.021	0	1
25	Pea	E	0.849	0.151	0.73	0	0.001	0.05	0.218	0	1
26	Pea	E	0.79	0.21	0.299	0	0.042	0.155	0.499	0.006	1.167
27	Pea	E[a]	0.012	0.988[b]	0	0.981	0	0.001	0	0.019	0.667
28	Pea	E	0.917	0.083	0.952	0	0	0.008	0.04	0	1.333
29	Pea	E	0.773	0.228	0.79	0	0	0.063	0.147	0	1
30	Pea	E	0.224	0.776	0	0.282	0.042	0.203	0.011	0.463	0.333
31	Pea	B	0.136	0.865	0	0.668	0	0.07	0	0.262	0.333
32	Pea	E	0.994[b]	0.006	0.998[b]	0	0	0	0.002	0	1.333
33	Pea	E	0.668	0.332	0.063	0	0.141	0.251	0.521	0.024	0.333
34	Pea	B	0.006	0.994[b]	0	0.997[b]	0	0	0	0.003	0
35	Pea	B	0.006	0.994[b]	0	0.995[b]	0	0	0	0.005	0.333
36	Pea	B	0.046	0.954[b]	0	0.941	0	0.011	0	0.049	0.333
37	Pea	E[a]	0.057	0.944[b]	0	0.871	0	0.016	0	0.113	0.667
38	Pea	E	0.776	0.224	0.803	0	0.001	0.048	0.147	0	1
39	Choctaw.	B	0.006	0.994[b]	0	0.996[b]	0	0	0	0.004	0
142	Choctaw.	E[a]	0.036	0.964[b]	0	0.917	0	0.009	0	0.074	0.833
143	Choctaw.	E[a]	0.005	0.995[b]	0	0.997[b]	0	0	0	0.003	0
144	Choctaw.	E[a]	0.063	0.938[b]	0	0.775	0.001	0.026	0	0.199	0
145	Choctaw.	B	0.108	0.892	0	0.728	0.001	0.067	0	0.204	0
146	Choctaw.	B	0.006	0.994[b]	0	0.995[b]	0	0	0	0.005	0.833
147	Choctaw.	B	0.006	0.994[b]	0	0.996[b]	0	0	0	0.004	0
148	Choctaw.	B	0.006	0.994[b]	0	0.996[b]	0	0	0	0.004	0
149	Choctaw.	B	0.009	0.991[b]	0	0.993[b]	0	0	0	0.007	0
CJL-51	Choctaw.	B	0.015	0.985[b]	0	0.97	0	0.002	0	0.029	—
CJL-81	Choctaw.	B	0.012	0.988[b]	0	0.981	0	0.002	0	0.017	—
CJL-82	Choctaw.	B	0.005	0.995[b]	0	0.996[b]	0	0	0	0.004	—
CJL-84	Choctaw.	B	0.101	0.899	0	0.867	0	0.044	0	0.089	—
86	Ichaway.	B	0.006	0.994[b]	0	0.996	0	0	0	0.004	0
87	Ichaway.	B	0.009	0.991[b]	0	0.988	0	0.001	0	0.011	0
88	Ichaway.	B	0.012	0.988[b]	0	0.978	0	0.002	0	0.02	0
89	Ichaway.	B	0.008	0.992[b]	0	0.99	0	0.001	0	0.009	—
90	Ichaway.	B	0.005	0.995[b]	0	0.997	0	0	0	0.002	0
93	Ichaway.	B	0.007	0.993[b]	0	0.994	0	0	0	0.006	0.4
94	Ichaway.	B	0.005	0.995[b]	0	0.996	0	0	0	0.003	0
96	Ichaway.	B	0.023	0.977[b]	0	0.939	0	0.004	0	0.056	0
97	Ichaway.	B	0.005	0.995[b]	0	0.996	0	0	0	0.003	0
98	Ichaway.	B	0.005	0.995[b]	0	0.998	0	0	0	0.002	—
99	Ichaway.	B	0.009	0.991[b]	0	0.99	0	0.001	0	0.01	—
100	Ichaway.	B	0.016	0.984[b]	0	0.963	0	0.002	0	0.035	0
101	Ichaway.	B	0.005	0.995[b]	0	0.998	0	0	0	0.002	0
103	Ichaway.	B	0.012	0.988[b]	0	0.977	0	0.003	0	0.021	—

Appendix 1. Continued.

			STRUCTURE		NewHybrids						
Code	River	mtDNA	E	B	E	B	F1	F2	E-BC	B-BC	MHI
104	Ichaway.	B	0.006	0.994[b]	0	0.996	0	0	0	0.003	—
105	Ichaway.	B	0.017	0.983[b]	0	0.984	0	0.001	0	0.016	0
106	Ichaway.	B	0.008	0.992[b]	0	0.991	0	0.001	0	0.008	0.4
111	Ichaway.	B	0.005	0.995[b]	0	0.996	0	0	0	0.003	0
112	Ichaway.	B	0.006	0.994[b]	0	0.995	0	0	0	0.005	0
113	Ichaway.	B	0.005	0.995[b]	0	0.997	0	0	0	0.003	0
114	Ichaway.	B	0.011	0.989[b]	0	0.978	0	0.003	0	0.019	0
116	Ichaway.	B	0.008	0.992[b]	0	0.993	0	0	0	0.006	0
117	Ichaway.	B	0.006	0.994[b]	0	0.996	0	0	0	0.004	0
118	Ichaway.	B	0.009	0.991[b]	0	0.993	0	0	0	0.007	—
119	Ichaway.	B	0.01	0.99[b]	0	0.988	0	0.001	0	0.011	—
120	Ichaway.	B	0.027	0.973[b]	0	0.982	0	0.002	0	0.016	—
121	Ichaway.	B	0.007	0.993[b]	0	0.994	0	0	0	0.006	—
122	Ichaway.	B	0.006	0.994[b]	0	0.996	0	0	0	0.004	—
123	Ichaway.	B	0.005	0.995[b]	0	0.997	0	0	0	0.003	0
124	Ichaway.	B	0.006	0.994[b]	0	0.997	0	0	0	0.003	—

[a] Haplotypes that disagree with the classification based on the STRUCTURE analysis of the microsatellite data.
[b] Values exceed the assignment thresholds established via simulation.

Appendix 2. The number of individuals (*n*) from each site genotyped at a given locus and their allele frequencies.

Locus	Allele size/*n*	Conecuh	Yellow	Pea	Choctaw.	Ichaway.
TerpSH1	*n*	33	27	27	13	29
	242	0.015	0	0	0	0
	258	0.242	0.333	0.241	0.231	0.034
	262	0.091	0.019	0.204	0	0.034
	268	0.015	0.333	0	0	0.017
	272	0.318	0.241	0.296	0.692	0.362
	276	0.258	0.074	0.037	0.038	0.293
	280	0.03	0	0.13	0	0.034
	284	0	0	0	0	0.052
	288	0.015	0	0.093	0.038	0.138
	292	0.015	0	0	0	0
	296	0	0	0	0	0.034
TerpSH2	*n*	33	27	29	13	30
	167	1	1	0.569	0.038	0
	171	0	0	0.259	0.577	0.533
	175	0	0	0.017	0.038	0.017
	179	0	0	0.155	0.346	0.45
TerpSH5	*n*	33	27	29	12	30
	148	0	0	0.241	0.417	0.267
	152	0	0	0.121	0.083	0.333
	156	0.015	0	0	0	0.05
	160	0.167	0	0.086	0.083	0.067
	164	0.303	0.852	0.086	0	0
	168	0.03	0.093	0.034	0.333	0.033
	172	0.03	0	0	0	0.1
	176	0	0	0	0	0.083
	180	0.318	0.037	0.103	0.083	0.067
	184	0.121	0.019	0.31	0	0
	188	0	0	0.017	0	0
	192	0.015	0	0	0	0
GmuB08	*n*	33	27	28	13	30
	232	0	0	0.411	0.654	0.4
	238	0	0	0	0.077	0.033
	241	0.015	0	0.071	0.077	0.317
	244	0	0	0	0.077	0.217
	247	0.652	0.944	0.393	0.077	0.033
	250	0.333	0.056	0.125	0.038	0
GmuD51	*n*	33	27	29	12	30
	281	0	0	0	0	0.067
	285	0	0	0.086	0.25	0.083
	289	0	0	0	0	0.133
	293	0	0	0.103	0.25	0.183
	297	0	0	0.034	0.083	0.1
	301	0	0	0	0.042	0.117
	305	0	0	0.103	0.25	0.233
	309	0	0	0.034	0.042	0.017
	313	0	0	0	0	0.05
	345	0	0	0	0	0.017
	349	0.045	0	0.121	0.042	0
	353	0.197	0.148	0	0.042	0
	357	0.242	0.13	0.138	0	0
	361	0.197	0.5	0.103	0	0
	365	0.136	0.148	0.19	0	0
	369	0.136	0.074	0	0	0
	373	0.045	0	0.086	0	0
GmuD70	*n*	33	26	29	11	30
	205	0	0	0	0	0.033
	213	0	0	0.121	0.227	0.117
	217	0	0	0.241	0.455	0.317
	221	0	0	0	0	0.017
	225	0.106	0.019	0.224	0.136	0.283

Appendix 2. Continued.

Locus	Allele size/*n*	Conecuh	Yellow	Pea	Choctaw.	Ichaway.
	229	0.045	0	0.103	0.045	0
	233	0.106	0	0.103	0.045	0
	237	0.333	0.5	0.034	0	0
	241	0.318	0.346	0.069	0	0.133
	245	0.091	0.135	0	0	0.017
	257	0	0	0	0	0.017
	261	0	0	0.103	0.091	0.067