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RESEARCH ARTICLE

Conservation genetics of the alligator snapping turtle: cytonuclear evidence of range-wide bottleneck effects and unusually pronounced geographic structure

A. A. Echelle · J. C. Hackler · J. B. Lack · S. R. Ballard · J. Roman · S. F. Fox · D. M. Leslie Jr. · R. A. Van Den Bussche

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Abstract A previous mtDNA study indicated that female-mediated gene flow was extremely rare among alligator snapping turtle populations in different drainages of the Gulf of Mexico. In this study, we used variation at seven microsatellite DNA loci to assess the possibility of male-mediated gene flow, we augmented the mtDNA survey with additional sampling of the large Mississippi River System, and we evaluated the hypothesis that the consistently low within-population mtDNA diversity reflects past population bottlenecks. The results show that dispersal between drainages of the Gulf of Mexico is rare ($F_{STmsat} = 0.43$, $\Phi_{STmtDNA} = 0.98$). Past range-wide bottlenecks are indicated by several genetic signals, including low diversity for microsatellites (1.1–3.9 alleles/locus; $H_e = 0.06$–0.53) and mtDNA ($h = 0.00$ for most drainages; $\pi = 0.000$–0.001). Microsatellite data reinforce the conclusion from mtDNA that the Suwannee River population might eventually be recognized as a distinct taxonomic unit. It was the only population showing fixation or near fixation for otherwise rare microsatellite alleles. Six evolutionarily significant units are recommended on the basis of reciprocal mtDNA monophyly and high levels of microsatellite DNA divergence.

Keywords Alligator snapping turtle · *Macrochelys* · mtDNA · Microsatellite DNA · Conservation genetics

A. A. Echelle (✉) · J. C. Hackler · J. B. Lack · S. F. Fox · R. A. Van Den Bussche
Department of Zoology, Oklahoma State University, Stillwater, OK 74078, USA
e-mail: anthony.echelle@okstate.edu

S. R. Ballard
Illinois Department of Natural Resources, Division of Natural Heritage, 9053 Route 148, Marion, IL, USA

J. Roman
Gund Institute for Ecological Economics, University of Vermont, Burlington, VT, USA

D. M. Leslie Jr.
Oklahoma Cooperative Fish and Wildlife Research Unit, US Geological Survey, Oklahoma State University, Stillwater, OK, USA

Introduction

Many studies demonstrate the value of examining both nuclear DNA and mitochondrial DNA (mtDNA) in assessing genetic structure for conservation management purposes. For example, Bowen et al. (1992) invoked female philopatry to explain significant mtDNA haplotype frequency differences among green sea turtles from different nesting beaches. Subsequent analysis of biparentally inherited nuclear markers revealed male-mediated gene flow and a level of population connectedness that was highly relevant to conservation of the species (Fitzsimmons et al. 1997; Roberts et al. 2004; Bowen et al. 2005). In this paper, we use variation in nuclear microsatellite DNA and additional sampling of mtDNA to re-assess the conservation genetics implications of an earlier mtDNA survey (Roman et al. 1999) of the alligator snapping turtle (*Macrochelys temminckii*), a large (up to 100 kg), riverine species in drainages of the Gulf of Mexico in southeastern United States.

Our primary objective is to provide a nuclear DNA perspective on population structure and number of evolutionarily significant units (ESUs) across the geographic range of the alligator snapping turtle. Identification of ESUs allows conservation managers to focus on populations harboring unique aspects of the evolutionary legacy

of a species, thereby promoting maintenance of evolutionary potential (Waples 1991; Moritz 1994). The most widely used frameworks for ESU recognition include two requiring some degree of genetic and adaptive distinctiveness (Waples 1991; Crandall et al. 2000) and one based only on genetic characters, specifically reciprocal monophyly for mtDNA and significant divergence in nuclear allele frequencies (Moritz 1994). For alligator snapping turtles, there is strong geographic structure in mtDNA variation but little basis for using adaptive traits in identifying ESUs (Roman et al. 1999). Our purpose was to add nuclear genetic data and apply Moritz's (1994) criteria for ESU recognition.

Based on mtDNA results alone, Roman et al. (1999) proposed three "major evolutionary lineages" as alligator snapping turtle ESUs: (1) a western assemblage in the Mississippi River and Gulf Coast streams from the Trinity River in east Texas eastward to Pensacola Bay of the Florida Panhandle, (2) a central assemblage extending across the rest of the Florida Panhandle, and (3) an eastern assemblage in the Suwannee River of Florida that might be considered a separate species based on level of genetic divergence. This was a conservative proposition, because the pattern of reciprocal monophyly in the mtDNA tree (Roman et al. 1999) is consistent with recognition of six ESUs: three within the western assemblage, two within the central assemblage, and one from the eastern assemblage (Suwannee River). A confounding factor for Moritz's (1994) approach to ESU recognition is that, because of high statistical power associated with microsatellites and other hypervariable markers, the detection of significant frequency differences might have little biological meaning (Hedrick 1999), resulting in over-recognition of ESUs. In our view, ESU recognition would be supported if nuclear markers suggest a history of isolation comparable to that indicated by reciprocal mtDNA monophyly.

Most populations within the ESUs proposed by Roman et al. (1999) carried haplotypes that were either endemic to their respective river system or shared only with populations in adjacent Gulf Coast drainages, indicating that female-mediated gene flow is extremely low ($\Phi_{ST} = 0.98$). The available data indicate that only nesting females leave the water and then only for a few meters where they nest and return to the water (Ernst et al. 1994). Although primarily freshwater, the species is occasionally found in brackish waters (Pritchard 1989, 2006) and it is possible that, via male-biased dispersal along coastal waterways, populations in different drainages of the Gulf of Mexico show greater connectedness than indicated by fixation for different mtDNA lineages.

Because of their large size (up to 100 kg) and susceptibility to trapping, alligator snapping turtles have long been harvested for meat, causing population declines throughout the range of the species (Pritchard 1989; Sloan and Lovich 1995; Conant and Collins 1998; Riedle et al. 2005). The World Conservation Union classified the species as Vulnerable and likely to become Endangered if factors leading to its decline continue (IUCN 2008). To improve monitoring and regulation of export, the United States Fish and Wildlife Service placed the species in Appendix III of the Convention on International Trade in Endangered Species (USFWS 2005). The alligator snapping turtle is not listed as threatened or endangered under the US Endangered Species Act, but it is treated as a species of conservation concern and afforded some protection by every state within its range (Pritchard 2006).

Our overall purpose is to help inform the conservation and management of alligator snapping turtles by addressing the following objectives: (1) to assess mtDNA diversity of previously unsurveyed populations in western (Oklahoma) and northern (Illinois, Missouri, and Tennessee) portions of the range, (2) to use microsatellite DNA variation to evaluate proposed ESUs, and (3) to test for evidence of male-mediated gene flow. We also use microsatellites to examine the possibility that the low levels of within-population mtDNA diversity observed by Roman et al. (1999) reflect past bottlenecks in population size.

Methods

Sampling

We obtained DNA or tissue samples from 196 alligator snapping turtles at localities encompassing much of the native geographic range (Fig. 1). DNA aliquots from 128 of the 158 turtles assayed by Roman et al. (1999) were available, and we used 67 additional turtles from the Mississippi River, five from museum collections and 62 collected in hoop-nets baited with fresh fish (Table 1; Appendix 1). Upon collection, we stored tissue (blood from the caudal vein or snips of tissue from the tail or rear-foot webbing) in 500 µl of lysis buffer (Longmire et al. 1997) and released the turtle at the site of capture.

For microsatellites, we assayed all 195 turtles for one trinucleotide (*Mte*A105) and six tetranucleotide (*Mte*C1, *Mte*C112, *Mte*D2, *Mte*D9, *Mte*D106, and *Mte*D109) loci described by Hackler et al. (2007). We PCR-amplified these loci in 15-µl reactions (9.0 µl ABI True Allele genotyping premix, 3.8 µl ddH_2O, 1.0 µl 5.9-µM primer pair, and 1.2 µl template DNA) with the following conditions: 95°C for 12 min; 35 cycles of 94°C for 40 s, 57°C for 40 s, and 72°C for 30 s; 72°C for 4 min. Then 1.5 µl of product was added to 3.5 µl of loading mix containing a size standard (ROX) and 1.5 µl of this mixture was analyzed using ABI's 3130 Genetic Analyzer and Genescan 3.1 to visualize

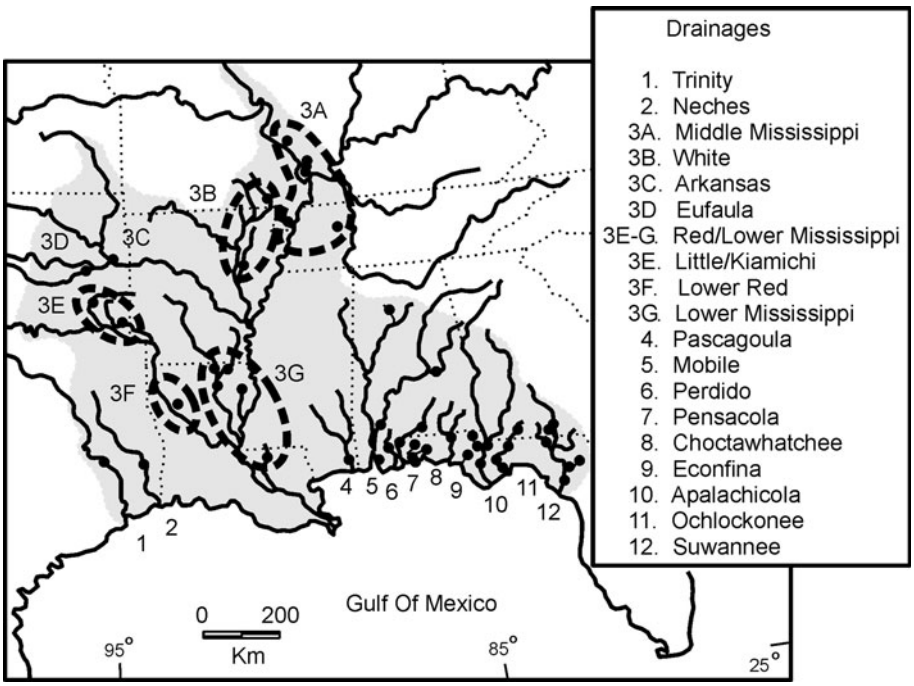

Fig. 1 Sampling locales for alligator snapping turtles. *Shaded area* approximate native range, *dots* trapping sites. Collections 3E, 3F, and 3G are combined as the Red River/ Louisiana population in some analyses

Table 1 Mitochondrial DNA haplotypes and microsatellite DNA assignments of alligator snapping turtles to populations with $n \geq 10$

Map number/Population	mtDNA		Microsatellite assignments												Percent correctly assigned
	Haplotype	*n*	2	3A	3B	3C	3D	3 E–G	4	5	7	10	11	12	
1 Trinity	A	3/0	3												–
2 Neches	A	18/0	10					1							91
3A Middle Mississippi	A	0/13		6	4		1	2							46
3B White	A	11/1		1	6	4		1							50
3C Arkansas	A	0/33			3	23	7								70
3D Canadian	A	0/10				2	8								80
3E Little and Kiamichi	A	0/5		1			2	3							–
3F Lower Red	A	0/4						4							–
3G Lower Mississippi	A	6/0			3			3							–
3E–G Red/Lower Mississippi	A	6/9		1	3		2	9							60
4 Pascagoula	B	13/0							13						100
5 Mobile	C, D	12/0								12					100
6 Perdido	C	1/0								1					–
7 Pensacola	E, F	23/0									20				100
8 Choctawhatchee	H	1/0										2			–
9 Econfina	J	8/0										2			–
10 Apalachicola	G, H, I	25/0										23			100
11 Ochlockonee	H	13/0											10		100
12 Suwannee	K	18/0												15	100

Population numbers are from Fig. 1; *n* = mtDNA sample sizes from Roman et al. (1999) and (right of slash) present study

microsatellites and Genotyper 2.5 or GeneMapper 3.7 to determine genotypes.

For mtDNA, we used primers from Roman et al. (1999) and PCR-amplified 420 base pairs, including the $tRNA^{Pro}$ and adjoining 5′ end of the control region, in 50-µl reaction volumes under the following conditions: 94°C for 3 min and 35 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min. The product was purified with the Wizard PCR Prep DNA Purification System (Promega Corporation, Madison, Wisconsin), and both strands were sequenced

using the aforementioned primers and either an Applied Biosystems Inc. (ABI) 377 Automated DNA Sequencer or ABI 3130 Genetic Analyzer. We used AssemblyLIGN 1.0.9 (Oxford Molecular Group PLC 1998) to assemble overlapping fragments within individuals and CLUSTAL X (Thompson et al. 1997) to align sequences, including each haplotype detected by Roman et al. (1999).

Analysis

Except for analyses of molecular variance (AMOVA; see below), we did not do standard genetic analyses on the mtDNA data because the results would have been identical to those of Roman et al. (1999). All turtles not in that study were from the Mississippi River basin, and, as in the previous report, they were fixed for haplotype A (Table 1).

We used GenAlEx (v6; Peakall and Smouse 2006) to compute expected and observed heterozygosity (H_e and H_o), number of alleles per locus (A), and number of private alleles (A_p). We used FSTAT (v2.9.3.2; Goudet 1995) to compute, for populations with $n \geq 10$, allele richness (A_r) corrected for sample size of 10. For exact tests of Hardy–Weinberg equilibrium (HWE) and pairwise linkage disequilibrium (10,000 iterations), we used GENEPOP on the web (http://genepop.curtin.edu.au/; Raymond and Rousett 1995). For exact tests we used the sequential Bonferroni (Rice 1989) correction to reduce Type I error for multiple comparisons ($\alpha = 0.05$). Instances of deviation from HWE were assessed for scoring errors and null alleles with Micro-Checker (Van Oosterhout et al. 2004). We used SPAGeDi (Hardy and Vekemans 2002) for an allele-size permutation test (5,000 iterations) to assess whether differences in microsatellite allele size (mutation) contributed to genetic divergence ($R_{ST} > F_{ST}$) or whether divergence is attributable to genetic drift alone.

We used Arlequin (v3.1; Excoffier et al. 2005) to compute analyses of molecular variance (AMOVAs; with both microsatellites and mtDNA) and pairwise F_{ST} values. For comparisons of mtDNA and microsatellite DNA indices we expressed Φ_{ST} and F_{ST} as standardized values (φ'_{ST}); i.e., observed values divided by the maximum possible, given the detected within-population variation (Hedrick 2005). To compute the maximum, we used Arlequin to perform AMOVAs with the data re-coded, as suggested by Meirmans (2006), to retain observed within-population diversity, but with no between-population sharing of alleles (or mtDNA haplotypes).

We employed three tests for past population bottlenecks. First, we used BOTTLENECK (v 1.2.02; Cornuet and Luikart 1996) with three different models of mutation, the stepwise mutation (SMM), infinite alleles (IAM), and a two-phase model (TPM) with 10% multistep and 90% single-step mutations, a reasonable ratio for microsatellites (Di Rienzo et al. 1994). The Wilcoxon signed-rank test was used to test for significant heterozygosity (H_e) excess over expectations from number of alleles present and mutation/drift equilibrium. In a second test, BOTTLENECK provided an assessment of whether the expected distribution of allele-frequency classes conformed to expectations for mutation-drift equilibrium. Past bottlenecks are indicated when the rare frequency-class (0.00–0.10) is less common than other classes (Luikart et al. 1998).

In the third test for bottlenecks, we used AGARst (v. 3.3; E. H. Harley, pers. comm.) to compute M (Garza and Williamson 2001), the mean ratio across loci, of number of alleles detected to the maximum possible (under SMM) for the observed allele size-range (Garza and Williamson 2001). We used M-crit (Garza and Williamson 2001; http://swfsc.noaa.gov/textblock.aspx?Division=FED&id=3298) to find the critical value M_c, below which bottlenecks are inferred. Inputs for each population were sample size, number of polymorphic loci, Θ ($=4N_e\mu$; N_e = effective population size, μ = mutation rate), and, from a literature survey by Garza and Williamson (2001), average proportion (0.12) and size (2.8) of non-one-step mutations. We computed Θ using the coalescence approach in Migrate (v2.4.4; Beerli and Felsenstein 1999) with the following settings: Bayesian inference, Metropolis-Hastings sampling, ladder (stepwise) model for microsatellite data, heating with four chains (1.0 1.5, 3.0, and 6.0). The resulting Θ for each population is the mean from two runs, each with 500 000 MCMC steps (burn in = first 10^4).

To explore the genetic effects of a population bottleneck in alligator snapping turtles we used BottleSim (Kuo and Janzen 2003), which is specifically designed to simulate effects on long-lived species. To represent the pre-bottleneck population, we used a microsatellite dataset (11 loci, 1–11 alleles/locus, 73 turtles) from a Nebraska population of ornate box turtle showing no evidence of a past bottleneck (Kuo and Janzen 2004). The average number of microsatellite alleles per locus (8.7) was near that (8.2) reported in a review of turtle population genetics (Fitz-Simmons and Hart 2007). The user input consisted of the genotype for each of the 73 turtles in the dataset, number of iterations (500), number of bottleneck years (300) and various demographic parameters: overlap in generations (100%), expected longevity (40 years in the wild; Dobie 1971), age at first reproduction (14; Pritchard 2006), sex ratio in the starting population (1:1; Riedle et al. 2008), and population size for each simulated year ($N = 50$, 100, or 200 in separate runs; 500 for the pre-bottleneck population). We used the BottleSim output for average number of alleles per locus in each succeeding year.

Using POPULATIONS (v1.2.30; http://bioinformatics.org/~tryphon/populations), we constructed neighbor-joining trees (mid-point rooting) and computed nodal support

(bootstrapped across loci; 10,000 replicates) from pairwise F_{ST} and Cavalli Sforza–Edwards chord distances (D_C). To visualize genetic variation within and between populations, we used GenAlEx to perform a principal coordinates analysis of genetic distances among individual genotypes (Smouse and Peakall 1999).

We used GeneClass2 (Piry et al. 2004) with the leave-one-out option and the Bayesian MCMC re-sampling method for allele frequencies (Rannala and Mountain 1997) to assess the most likely population assignment for all 188 turtles from the 12 populations with $n \geq 10$. To search for hybrids and first-generation immigrants among those 12 populations, we used Structure (v2.2; Pritchard et al. 2000) for a Bayesian assessment of ancestry for individual turtles. Parameters were $K = 12$, generations back = 3, correlated allele frequencies, and 1.5×10^6 MCMC iterations (burnin = 1.0×10^6). Each turtle was given prior population assignment based on drainage or area (Fig. 1) of collection, allowing posterior probability estimates (q) of whether it was (1) a member of the assigned population, (2) an immigrant from another population, (3) an F_1 hybrid with a parent from another population, or (4) a product of backcrossing two or more generations back. Two separate runs gave nearly identical results.

Results

As previously mentioned, the 67 mtDNA samples we added to this study were from the Mississippi River Basin. All were represented by haplotype A, which Roman et al. (1999) found to be fixed in this basin and the next two Gulf Coast drainages to the west (Trinity and Neches).

Numbers of alleles for the seven microsatellite loci were 3 (*Mte*D2 and *Mte*C112), 4 (*Mte*A105), 7 (*Mte*C1 and *Mte*D106), 11 (*Mte*D9), and 15 (*Mte*D109). The only evidence of HWE deviation involved *Mte*D106 heterozygote deficiencies in the Mobile and Apalachicola populations ($P < 0.00001$). The estimated null-allele frequency was 0.19 for the 12 turtles from Mobile Bay and 0.29 for the 23 from Apalachicola, giving expectations of, respectively, 0.4 and 1.9 *Mte*D106 null homozygotes for a total of 2.3 from the two localities. There were, however, no instances of non-amplification in these collections, nor in any other turtles except two from Illinois. The homozygote excess in Mobile Bay and Apalachicola probably reflects non-random sampling of families (e.g., siblings from heterozygous parents) rather than null alleles. There were no other indications of significant HWE deviation. Significant linkage disequilibrium among loci was detected only for the *Mte*D9/*Mte*D106 combination in the Arkansas River

Table 2 Genetic variation in populations of alligator snapping turtles (map numbers as in Fig. 1)

Map number/population	Microsatellite DNA						mtDNA	
	Number of polymorphic loci	A	A_r	A_p	H_e	H_o	h	π (%)
1 Trinity	5	2.0	–	1	0.29	0.29	0.00	0.000
2 Neches	6	3.0	2.8	0	0.47	0.47	0.00	0.000
3A Middle Mississippi	7	3.1	3.1	0	0.53	0.46	0.00	0.000
3B White	7	2.9	2.8	0	0.44	0.47	0.00	0.000
3C Arkansas	7	2.7	2.4	0	0.45	0.39	0.00	0.000
3D Canadian	7	3.0	3.2	0	0.43	0.37	0.00	0.000
3E Little and Kiamichi	7	3.1	–	0	0.48	0.41	0.00	0.000
3F Lower Red	7	2.7	–	0	0.46	0.54	0.00	0.000
3G Lower Mississippi	7	2.6	–	0	0.43	0.49	0.00	0.000
4 Pascagoula	5	3.4	3.2	3	0.39	0.35	0.00	0.000
5 Mobile	6	2.7	2.6	0	0.29	0.26	0.41	0.098
7 Pensacola	6	3.3	2.9	1	0.39	0.38	0.50	0.120
8 Choctawhatchee	3	1.7	–	0	0.29	0.36	0.00	0.000
9 Econfina	1	1.1	–	0	0.07	0.00	0.00	0.000
10 Apalachicola	6	3.9	3.3	5	0.44	0.35	0.29	0.072
11 Ochlockonee	3	1.7	1.7	0	0.26	0.30	0.00	0.000
12 Suwannee	3	1.4	1.3	0	0.06	0.07	0.00	0.000

A = mean number of alleles per locus, A_r = mean allele richness corrected for $n = 10$ (not calculated for collections with $n < 10$), and A_p = number of private alleles; H_e = mean expected heterozygosity, and H_o = mean observed heterozygosity. The mtDNA values are from Roman et al. (1999); h = haplotype diversity; π = nucleotide diversity. The one specimen from Perdido Bay (locality 6) is not included

population ($P < 0.00001$), possibly as a result of combining turtles from local populations having different allele frequencies.

Within-population diversity

Diversity was low for both mtDNA and microsatellites, with little correspondence between indices of variation for the two sets of data (Table 2). All except three populations (Mobile, Pensacola, and Apalachicola) had zero diversity for mtDNA, and nucleotide diversity (π) in the three exceptions was 0.1%. Among populations with 10 or more turtles, the two easternmost, Ochlockonee and Suwannee, had the lowest microsatellite diversity, with allele richness ($A_r = 1.7$ and 1.3) and expected heterozygosity ($H_e = 0.26$ and 0.06) well below two standard deviations from the mean for the rest of the populations ($A_r = 2.9 \pm 0.6$, $H_e = 0.42 \pm 0.14$).

Population clusters and ancestry analysis

The first three principal coordinate axes (PCO1-3) explained 71.2% of the variation among multilocus genotypes. Scores on PCO1 tended to separate western populations from eastern populations, with the Suwannee population as a distinct, non-overlapping cluster at the extreme of the eastern distribution (Fig. 3). The PCO1/PCO2 bi-plot further separated the turtles into two nearly non-overlapping groups, an eastern cluster from the Ochlockonee River to Perdido Bay, and a western cluster from Mobile Bay to the Trinity River (Fig. 2). Scores on PCO3 tended to separate populations in different eastern drainages (exclusive of the Suwannee) from one another, with an east-to-west tendency for increasing scores (Fig. 2). Populations from the Mississippi River basin showed broad overlap with each other and with scores from the Trinity and Neches river populations.

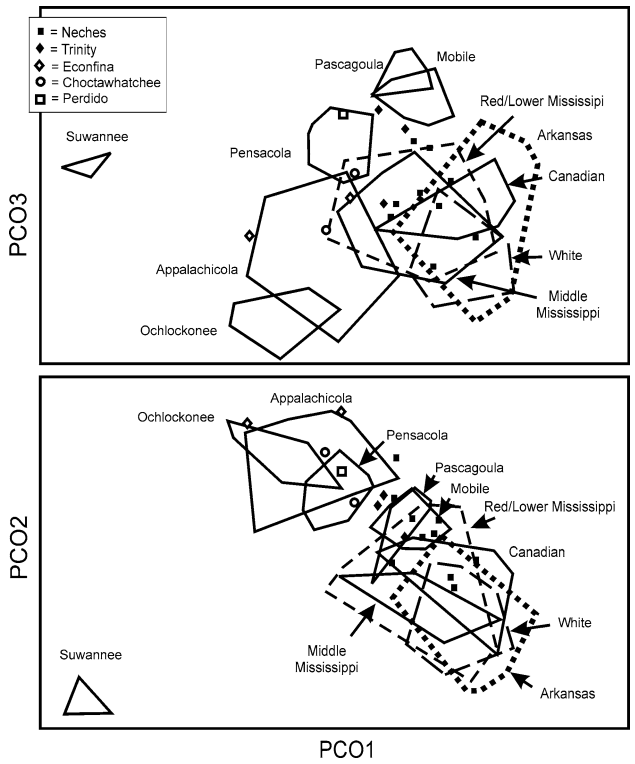

Fig. 2 Plots of principle coordinate scores for 196 alligator snapping turtles. *Polygons* maximum area polygons for each group. *Group labels* refer to drainages as in Fig. 1

GENECLASS placed turtles from six of the seven populations in the Mississippi River drainage (3A-G) in more than one population from the drainage (Table 1). All other turtles, which were lumped as a single population per Gulf of Mexico drainage, were, with one exception, assigned to the drainage of collection. The exception was a turtle from the Neches River that grouped most closely with the population from the Lower Mississippi River system (Red River/Louisiana). However, in the ancestry analysis from STRUCTURE, this turtle was assigned to the Neches population at $q = 0.94$.

In the ancestry analysis, all 188 turtles from the 12 populations with $n \geq 10$ had their highest probability of assignment to the population of capture. All except four of these had posterior probabilities (q) ≥ 0.70 for the population of collection and 94% (176) had values >0.90. The four with the lowest probabilities for the population of capture included three from the Mississippi River basin. These three included one from the White River ($q = 0.66$) with a low probability (0.12) of ancestry in the Lower Mississippi and two from the Canadian River (both with $q = 0.51$) with evidence of ancestry in the nearby Arkansas River, one having a low probability (0.14) of being a first-generation hybrid and one being a possible first-generation immigrant ($q = 0.37$). The only evidence of potential exchange between Gulf Coast drainages was an Apalachicola turtle ($q = 0.38$) with a probability of 0.27 of having an ancestor two more generations back in the Pensacola population ($q = 0.11$ and 0.16 for 2 and 3 generations back).

Distribution of microsatellite diversity

The results reported here are based on allele frequencies, not allele size, because there was no significant difference between global R_{ST} and F_{ST} values ($P = 0.33$). All but two of the 66 pairwise F_{ST}-values (summarized in Fig. 3) between populations with $n \geq 10$ were significant at $P < 0.05$. Six were not significant after Bonferroni correction (critical $P = 0.0008$), all of which were among the 10 comparisons within the Mississippi River System. The remainder, all comparing different drainages of the Gulf of

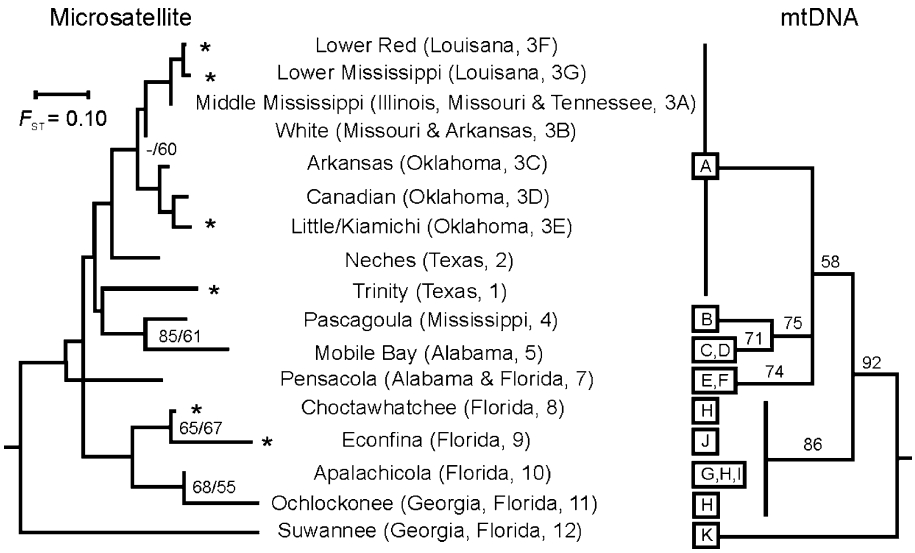

Fig. 3 Neighbor-joining tree based on microsatellite DNA variation and maximum parsimony tree for mtDNA haplotypes in alligator snapping turtles. Drainage names and locality numbers are from Fig. 1. The mtDNA tree is modified from Roman et al. (1999); letters in *rectangles* haplotypes, *numbers* on nodes show bootstrap values >50%. The microsatellite tree (with mid-point rooting) is based on the pairwise-F_{ST} matrix; *numbers* left of the slash = bootstrap support from F_{ST}; right of the slash = bootstrap support from chord distances (D_C). Terminal nodes marked with an *asterisk* had microsatellite sample sizes of <10 turtles (n = 2–6)

Table 3 AMOVA results for various groupings of alligator snapping turtles

Groupings	Percent of diversity attributable to differences				φ'_{ST}
	Among groups	Among populations within groups	Among populations	Within populations	
Microsatellite DNA					
Mississippi only	–	–	9.7*	90.3	0.19
Western only	–	–	29.8*	70.2	0.55
Eastern only	–	–	57.7*	42.3	0.81
Western vs eastern	4.7*	40.7*	45.4*	54.6	0.83
All populations	–	–	42.6*	57.4	0.76
mtDNA					
Mississippi only	na	na	na	na	na
Western only	–	–	96.0*	4.0	1.02
Eastern only	–	–	97.4*	2.6	0.99
Western vs eastern	24.4*	72.5*	96.9*	2.1	0.99
All populations	–	–	97.8*	2.3	1.00

Except for "Mississippi only" (=populations 3A–3G), all "populations" are the composite samples from each drainage of the Gulf of Mexico (Mississippi River samples lumped as one), and only the eight drainages with $n \geq 10$ are considered. * Significance at $P < 0.00001$; na = not applicable because of zero variation. Results are based on F_{ST} for microsatellite DNA and Φ_{ST} for mtDNA; φ'_{ST} = ratio of observed F_{ST} (or Φ_{ST}) to the maximum possible with the observed within-population variation (Meirmans 2006)

Mexico, ranged from F_{ST} = 0.16–0.81. The lowest inter-drainage values were between the Neches and the Mississippi River system (minimum = 0.016 for the Neches vs. Red River/Louisiana comparison, others = 0.27–0.33). The remaining comparisons ranged from F_{ST} = 0.34 (Mobile vs. Pascagoula) to F_{ST} = 0.81 (Ochlockonee vs. Suwannee). The Suwannee was the most divergent, with F_{ST} values of 0.66–0.81.

The AMOVA for microsatellites in turtles from the eight drainages with $n \geq 10$ attributed 57.4% of total diversity to variation within drainages, whereas this component was only 2.3% for mtDNA (Table 3). Correspondingly, 42.6 and 97.8% of the diversity was attributable to differences among drainages. When stratified into eastern (Apalachicola to Suwannee) and western (Neches to Pensacola) groups, the among-group component accounted for only

4.7% of the microsatellite diversity, but accounted for 24.5% of the mtDNA diversity. The among-drainages-within-groups component accounted for 40.7 and 72.5%, respectively, of the microsatellite and mtDNA diversity. For the western group, the within-drainage component was greater than the among-drainage component (70.2 vs. 29.8%), whereas this was reversed for the eastern group (42.3 vs. 57.7%). For mtDNA, 96.0% of western and 97.4% of eastern diversity was attributable to differences among drainages. Within the Mississippi River system, most microsatellite variation (90.3%) was attributable to within-population variation; mtDNA diversity was zero.

The standardized index of divergence φ'_{ST} was greater for mtDNA than for microsatellites (Table 3). The mtDNA values were around the maximum (1.00) for all comparisons, whereas for microsatellites they were 0.55 and 0.81 for, respectively, western and eastern populations, and 0.76 across all populations. For Mississippi River populations only, the microsatellite φ'_{ST} was 0.19.

Tests for past bottlenecks

Ten of the 12 populations with $n \geq 10$ had *M*-ratios lower than the critical value (M_c), indicating past bottlenecks in population size (Table 4). Four populations with depressed *M*-ratios also showed significant (Middle Mississippi and Arkansas River; $P = 0.004$–0.027) or nearly significant (Apalachicola and Ochlockonee; $P = 0.055$–0.063) excesses of heterozygosity relative to expectations from mutation/drift equilibrium, and three of those four showed the modal shift in allele frequency classes expected from past population reductions (Table 4).

Bottleneck simulations

Starting with the genetic structure of a natural population showing no evidence of previous bottlenecks, and assuming bottleneck population size $N = 50$, we obtained the following percentages for number of alleles remaining after different intervals of time: 79% (50 years), 72% (100 years), 61% (200 years), and 54% (300 years). The corresponding percentages for $N = 100$ were 90, 83, 75, and 68%, and those for $N = 200$ were 95, 92, 85, and 81%.

Relationships among populations

The microsatellite trees based on F_{ST} (Fig. 3) and D_C (not shown) differed only in minor ways. Both indicated a group comprising all populations from the Mississippi River system (with bootstrap support in the D_C tree). With two exceptions, namely placement (no bootstrap support) of the Trinity and Pensacola populations, both trees were consistent with the supported nodes in the mtDNA tree. The microsatellite placement of the Trinity population probably is a result of small sample size ($n = 3$), because in the assignment test it grouped with the Neches population, which is more likely based on geography. The mtDNA placement of Pensacola received only weak bootstrap support (58%). The microsatellite and mtDNA trees agree in grouping the small ($n = 2$) Choctawhatchee

Table 4 Results from three tests for past bottlenecks in all populations with $n \geq 10$

Population	Heterozygosity excess		Allele frequency shift	*M*-Test	
	Model showing significance	*P* (no excess)		M_c	*M*
Middle Mississippi	IAM	0.027	Yes	0.83(7)	0.72*
White	None	ns	Yes	0.83(7)	0.73*
Arkansas	IAM, TPM	0.004, 0.020	No	0.83(7)	0.64*
Canadian	None	ns	No	0.83(7)	0.68*
Red/Lower Mississippi	None	ns	No	0.83(7)	0.76*
Neches	None	ns	No	0.82(6)	0.80*
Pascagoula	None	ns	No	0.81(5)	0.81
Mobile	None	ns	No	0.83(6)	0.72*
Pensacola	None	ns	No	0.83(6)	0.73*
Apalachicola	IAM	0.055	No	0.83(6)	0.63*
Ochlockonee	IAM, TPM, SMM	0.063 (all 3)	Yes	0.78(3)	0.71*
Suwannee	None	ns	No	0.78(3)	1.00

IAM, SSM, and TPM = mutation models. Heterozygosity excess = excess based on mutation-drift equilibrium. The Red/Lower Mississippi population is the lumped composite of collections 3E, 3F, and 3G (Fig. 1). All *P*-values <0.10 are shown; ns = $P > 0.10$. Parentheses = number of loci contributing to the *M*-test (monomorphic loci do not contribute)

* *M* smaller than M_c, indicating past bottlenecks

and Econfina samples with the Apalachicola and Ochlockonee samples. The Suwannee population was the most divergent for both datasets; its basal position in tree topology received high support (92%) from mtDNA, but the basal nodes of the microsatellite tree received no statistical support.

Discussion

Our conclusions from microsatellites are tempered by relatively small sample sizes and ascertainment bias due to non-random sampling, both of which reflect the scarcity and patchy distribution of the alligator snapping turtle. The overall relatively weak evidence of linkage disequilibrium and deviation from HWE indicates that the microsatellite results allow useful inferences on population structure, so long as over-interpretations are avoided. For example, we did not attempt to estimate effective population sizes from genotypic frequencies because the available approaches (e.g., Tallmon et al. 2008; Waples and Do 2008) are sensitive to effects such as minor, statistically insignificant deviations from HWE and linkage disequilibrium due to non-random sampling.

The mtDNA and nuclear DNA data together indicate that genetic exchange must be extremely rare among populations of alligator snapping turtles in different drainages of the Gulf of Mexico. For mtDNA, 98% of total diversity reflected differences among drainages, which might be the highest value reported for conspecific populations (Roman et al. 1999). The standardized index φ'_{ST} across all drainages was around 1.00 for mtDNA and 0.76 for nuclear DNA. The nuclear DNA value, although lower than for mtDNA, is large and consistent with expectations based on an absence of male-mediated gene flow. Genetic drift, and the associated proportion of diversity attributable to differences among populations, should be greater for mtDNA because its uniparental, effectively haploid inheritance results in effective population sizes about four times smaller than for nuclear DNA. For microsatellites, the pairwise percentages of diversity associated with between-drainage differences ($100*F_{ST}$) were 27–81% except for Neches versus Red River/Louisiana (16%). Values greater than 20% correspond to long-term migration rates of less than one individual per generation under selective neutrality and migration-drift equilibrium (Hedrick 2000). Such rates have negligible effect on frequencies of genetic markers (Mills and Allendorf 1996).

Several observations indicate that the alligator snapping turtle might have experienced past bottlenecks over its entire geographic range. First, diversity for both mtDNA and microsatellites is extremely low. For mtDNA, the populations in most of the different drainages of the Gulf of Mexico are fixed for a single, regionally endemic haplotype. In the three instances of polymorphism, the indexes of diversity are low ($h \leq 0.50$; $\pi < 0.001$) and consistent with expectation from past bottlenecks (Grant and Bowen 1998). For microsatellites, number of alleles per locus per population was much lower than reported in a comparable study of nine populations ($n = 11$–22; 5 loci) of diamondback terrapin (1.4–3.9 vs. 6.5–12.7; Hauswaldt and Glenn 2005) and well below the average (8.2; range = 1.5–18.0) in a review of turtle microsatellite DNA studies (FitzSimmons and Hart 2007). The range for alligator snapping turtles is well within that reported for populations showing bottleneck effects, including the review by Garza and Williamson (2001; 2.1–5.3; 7 mammals, 1 fish), and a more recent report for trumpeter swan (2.4–3.2; Oyler-McCance et al. 2007). Finally, the *M*-test detected potential past bottlenecks for 10 of 12 tested populations of alligator snapping turtle, and five populations showed evidence of past bottlenecks on the basis of at least two of the three tests (*M*-test, heterozygosity excess, allele size-class frequency).

Some of our failures to obtain significant evidence of past bottlenecks might reflect low statistical power because of sample sizes and numbers of loci entering the tests. This is particularly applicable to the Suwannee population, in which four of the seven loci were monomorphic. Such loci, although potentially reflecting past bottlenecks, do not contribute to any of the three tests. The opposite problem, false indication of past bottlenecks, can result from alleles missed because of sample size. However, this is more severe for populations that, unlike alligator snappers, have large numbers of alleles. Further, the tests are tolerant of some loss of rare alleles, as Garza and Williamson (2001) noted for the *M*-test. These considerations, together with low mtDNA and microsatellite diversity in all populations, indicate that population bottlenecks have played a significant role in the genetic history of the species. Bottleneck effects, together with restricted inter-drainage gene flow, would lead to reduced within-drainage diversity and increased between-drainage divergence (Hedrick 1999), thereby augmenting the proportions of mtDNA and microsatellite diversity attributable to differences among populations.

The range-wide evidence for bottleneck effects in the alligator snapping turtle could reflect ancient or relatively recent events, or both. The high among-drainage mtDNA diversity seems to exclude loss of diversity in an ancestral population and subsequent range expansion because this predicts low mtDNA diversity across the range of the species, as observed for the common snapping turtle (Walker et al. 1998) and the bog turtle (Rosenbaum et al. 2007). Other possibilities include (1) range-wide anthropogenic impacts, (2) non-anthropogenic environmental

deterioration (e.g., past climate change) for the species, or (3) any combination of these, perhaps at different times in the history of the species. Genetic evidence of population declines potentially attributable to overharvest and habitat alteration by humans is restricted to the populations showing signs of past bottlenecks on the basis of heterozygosity excess and/or shift in allele-frequency classes. The signals from these tests indicate relatively recent bottlenecks (Garza and Williamson 2001), and Spear et al. (2006) invoked temporally separated bottlenecks to explain the observation that (as in our study) such signals were less pervasive than those for the *M*-test in Yellowstone National Park populations of tiger salamander.

Recent anthropogenic factors probably do not explain the genetic evidence for range-wide bottleneck effects. Within a large system like the Mississippi, overharvest likely was patchy and recent bottlenecks should have left a landscape mosaic of allele/haplotype distributions. Instead, populations in the large Mississippi River System show a fairly uniform allele composition, a lack of private alleles, and fixation for the same mtDNA haplotype. Furthermore, most of the human impact occurred over the last 50–60 years (Pritchard 2006) and this timeframe, which equates to only 3–4 hatchling-to-adult generations, is unlikely to cause a severe loss of diversity in an animal that lives 50+ years. Correspondingly, our simulated alligator snapping turtle populations with $N = 50$, 100, and 200 retained 79, 90, and 95%, respectively, of the starting number of alleles after 50 years.

It seems likely that the evidence for past, range-wide bottlenecks reflects events occurring before the modern era of human impacts. The *M*-test signal can persist for hundreds of generations, depending on rates of mutation and population rebound (Garza and Williamson 2001). The time would be extended for the alligator snapping turtle compared with many other animals because of extended longevity (40+ years), the 13–16 years required for maturation (Pritchard 2006), and, potentially, by an unusually low mutation rate in turtles (Avise et al. 1992, but see Fitzsimmons 1998). These considerations indicate an available timeframe of at least the past several thousand years for the hypothesized range-wide bottleneck events. Such long-term persistence of genetic signals of past bottlenecks is indicated for another long-lived turtle, the Galapagos tortoise taxon *Geochelone nigra vandenburghi*, which appears to have retained reduced mtDNA diversity and, for microsatellites, the *M*-test and heterozygosity-excess signals of past bottlenecks for 88 ky (Beheregaray et al. 2003).

The most recent event of sufficient temporal and geographic scale to cause a range-wide bottleneck in alligator snapping turtles seems likely to be the Hypsithermal Interval $\sim$ 8.5–5.0 kya, which had widespread impacts on the distribution and genetic structure of terrestrial and aquatic vertebrates of North America (Phillips et al. 2000; Douglas et al. 2003). The last 1,500 years (6.5–5.0 kya) of the Hypsithermal was especially severe, with unusually high temperatures and several periods of up to 300 years duration when extreme dryness prevailed in the southern Great Plains and eastern United States (Driese et al. 2008). Alligator snapping turtles are known primarily from mesic, lowland areas (Shipman 1993; Riedle et al. 2005) and the species might have been particularly sensitive to the long, Middle Holocene intervals of severe drought.

Conservation implications

Roman et al. (1999) recognized three ESUs based on mtDNA variation in alligator snapping turtles: a western assemblage (Trinity River eastward to Pensacola Bay), a central assemblage (Choctawhatchee to Ochlockonee), and the Suwannee River population. However, relationships of the Pensacola Bay population are not well resolved, and it is effectively the evolutionary equivalent of the central assemblage and the two subgroups of the western assemblage, Trinity/Neches/Mississippi and Pascagoula/Mobile/Perdido populations. Adherence to Mortiz's (1994) criteria, reciprocal monophyly for mtDNA and significant differences in nuclear gene frequencies, would recognize six ESUs comprising populations in the following drainages: (1) Trinity, Neches, and Mississippi, (2) Pascagoula, (3) Mobile and Perdido, (4) Pensacola, (5) Choctawhatchee, Econfina, Apalachicola, and Ochlockonee, and (6) Suwannee. The evidence of strong nuclear DNA divergence among the proposed ESUs supports the indication from reciprocal mtDNA monophyly that they have a long history of independent evolution and should be treated separately in conservation management plans for the species.

The most distinctive population of alligator snapping turtle is the one in the Suwannee River. It is fixed for an mtDNA haplotype that apparently has been diverging from those in all other snapping turtle populations since the late Pliocene or early Pleistocene (Roman et al. 1999). Correspondingly, the population is also the most divergent in microsatellite allele frequencies, and, at two loci, it is fixed ($Mte\text{A}105^{175}$) or nearly fixed ($Mte\text{C}1^{139}$, frequency $= 0.97$) for alleles that were otherwise absent or very rare. No other population showed this level of distinctiveness for effectively unique alleles. The nearest possibility is the Pensacola population, which had, at a frequency of 0.76, an allele ($Mte\text{A}105^{166}$) that was absent except for a frequency of 0.04 in the Apalachicola population. It is worth noting that the Suwannee population of another obligatorily aquatic chelonian of freshwater rivers, the spiny softshell turtle, was the basal member of a widespread mtDNA clade sampled from an area encompassing most of the range of alligator snapping turtle (McGaugh et al. 2008).

Microsatellites indicate that most populations delimited in this study are divergent in allele frequencies, qualifying them as management units (*sensu* Moritz 1994) within their respective ESU. Most Gulf Coast drainages have not been adequately sampled for insight into within-drainage management units, but nearly all assayed populations within the best-sampled drainage, the Mississippi River system, were significantly divergent based on microsatellites. To avoid over- or under-recognition of management units, conservation managers should focus on maintenance of natural patterns of genetic connection among populations, thereby preserving the processes that maintain diversity and evolutionary potential (Crandall et al. 2000). For example, managers should consider ways of mitigating for, or removing, dispersal barriers such as the many dams and lock-and-dam systems on major waterways (Roman et al. 1999; Riedle et al. 2005).

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Appendix 1

Specimens assayed. Locality number and drainage name in bold (Fig. 1). Number of turtles assayed in parentheses (mtDNA/microsatellites). Museum sources are listed under the following acronyms: FMNH = Field Museum of Natural History, Chicago, ISM = Illinois State Museum, Springfield, UIMNH = University of Illinois Museum of Natural History, APU = Austin Peay State University, Tennessee.

1 Trinity River (3/3); Bedias Creek, Madison and Leon Counties, Texas (3/3). **2 Neches River** (18/11); Bingham Lake, Tyler County, Texas (18/11). **3A Middle Mississippi River** (13/13); Wolf Bayou Conservation Area, Pemiscott County, Missouri (7/7), FMNH 3234, Ohio River at Cairo, Alexander County, Illinois (1/1), UIMNH 33124, Mississippi River at Chester, Randolph County, Illinois (1/1), Wolf Creek, Jackson County, Illinois (1/1), ISM 689915, Dutch Creek, Union County, Illinois (1/1), APU #0-10,11, Kentucky Lake at Whiteoak Creek, Houston County, Tennessee (1/1), APU #1-3, Kentucky Lake at Tischel Bay, Stewart County, Tennessee (1/1). **3B White River** (12/12); Black River, Butler County, Missouri (11/11), Cache River, unknown county, Arkansas (1/1). **3C Arkansas River** (33/33); Little Vian Creek, Sequoyah County, Oklahoma (8/8), Big Vian Creek, Sequoyah County, Oklahoma (8/8), Hezekiah Creek, Sequoyah County, Oklahoma (4/4), Dirty Creek, Muskogee County, Oklahoma (12/12), Briar Creek, Haskell County, Oklahoma (1/1). **3D Canadian River** (10/10); Mill Creek, McIntosh County, Oklahoma (10). **3E Little and Kiamichi rivers** (6/6); Hugo Lake, Choctaw County, Oklahoma (1/1), Mill Creek, Pushmataha County, Oklahoma (1/1), Mud Creek, McCurtain County, Oklahoma (1/1) Forked Lake, McCurtain County, Oklahoma (3/3). **3F Lower Red River** (4/4); Coushatta Bayou, Red River Parish, Louisiana (4/4), **3G Lower Mississippi River** (13/6); Tensas River, Madison Parish, Louisiana (1/1), Bayou Gallion, Morehouse Parish, Louisiana (3/2,) Bayou Desiard, Ouachita Parish, Louisiana (5/2), Bayou D'Arbonne, Union Parish, Louisiana (1/1). **4 Pascagoula River** (13/13); Pascagoula River, Jackson County, Mississippi (13/3). **5 Mobile Bay** (12); Bear Creek, Baldwin County, Alabama (3/3), Southern Delta, Balwin County, Alabama, (2/2) Turkey Creek, Baldwin County, Alabama (5/5), Tallapoosa River, Macon County, Alabama (1/1), Lost Creek, Walker County, Alabama (1/1). **6 Perdido Bay** (1/1); Styx River, Baldwin County, Alabama (1/1). **7 Pensacola Bay** (23/20); Conecuh River, Covington-Crenshaw counties, Alabama (4/4), Escambia River, Escambia County, Alabama (5/4), Escambia River, Santa Rosa County, Florida (1/1), East Bay River, Okaloosa County, Florida (4/3), Shoal River, Okaloosa County, Florida (3/3), Yellow River, Okaloosa County, Florida (4/3), Blackwater River, Santa Rosa County, Florida (2/2). **8 Choctawhatchee River** (2/2); Holmes Creek, Washington County, Florida (2/2). **9 Econfina River** (8/2); Blue Springs, Washington County, Florida (8/2). **10 Apalachicola River** (25/23); Apalachicola River, Calhoun County, Florida (1/1), The Bayou, Calhoun County, Florida (15/15), Chipola River, Jackson County, Florida (9/7). **11 Ochlockonee River** (10/10); Ochlockonee River, Liberty County, Florida (5/5), Ochlockonee River, Leon County, Florida (2/2), Ochlockonee River, Wakulla County, Florida (3/3). **12 Suwannee River** (18/15); Withlacoochie River, Lowndes County, Georgia (1/0), Alapaha River, Lowndes County, Georgia (1/0), Santa Fe River, Alachua and Union counties, Florida (10/9),

New River, Union County, Florida (1/1), Suwannee River, Dixie County, Florida (3/3), Suwannee River, Suwannee County, Florida (2/2).

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