Misleading phylogenetic inferences based on single-exemplar sampling in the turtle genus *Pseudemys*

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ABSTRACT

Reconstructing species trees for clades containing weakly delimited or incorrectly identified taxa is one of the most serious challenges facing systematists because building phylogenetic trees is generally predicated on correctly identifying species membership for the terminals in an analysis. A common practice, particularly in large-scale phylogenetic analyses, is to use single-exemplar sampling under the implicit assumption that the resulting phylogenetic trees will be poorly supported if the sampled taxa are not good species. We examine this fundamental assumption in the North American turtle genus *Pseudemys*, a group of common, widely distributed freshwater turtles whose species boundaries and phylogenetic relationships have challenged systematists for over half a century. We sequenced 10 nuclear and three mitochondrial genes from the nine currently recognized species and subspecies of *Pseudemys* using geographically-widespread sampling of each taxon, and analyzed the resulting 86-individual data set using population-genetic and phylogenetic methods. We found little or no evidence supporting the division of *Pseudemys* into its currently recognized species/subspecies. Rather, our data strongly suggest that the group has been oversplit and contains fewer species than currently recognized. Even so, when we conducted 100 replicated, single-exemplar phylogenetic analyses of these same nine taxa, most Bayesian trees were well resolved, had high posterior probabilities, and yet returned completely conflicting topologies. These analyses suggest that phylogenetic analyses based on single-exemplar sampling may recover trees that depend on the individuals that are sampled, rather than the underlying species tree that systematists assume they are estimating. Our results clearly indicate that final resolution of *Pseudemys* will require an integrated analysis of morphology and historical biogeographic data coupled with extensive geographic sampling and large amounts of molecular data, and we do not recommend taxonomic changes based on our analyses. If our 100-tree resampling experiments generalize to other taxa, they suggest that single-exemplar phylogenies should be interpreted with caution, particularly for groups where species are shallowly diverged or inadequately delimited.

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1. Introduction

Molecular phylogenetics encompasses a wide range of evolutionary problems, from recovering the deepest nodes in the Tree of Life to delimiting recently derived species, and methodological progress has moved forward at both ends of this spectrum. However, taxa that fall between relatively well-differentiated phylogenetic lineages and potentially subdivided populations often remain problematic because of the stochastic nature of gene-tree coalescence, potential introgression, and low information content of molecular sequences (Degnan and Rosenberg, 2006; Hudson and Coyne, 2002; Maddison and Knowles, 2006; Moore, 1995). These problems pose a challenge for species delimitation and downstream species-tree reconstruction because species-delimitation methods often require the use of a fully resolved input species phylogeny (e.g. Knowles and Carstens, 2007; Yang and Rannala, 2010), while species-tree reconstruction models assume little or no horizontal gene flow and often require that individuals be assigned to species *a priori* (Heled and Drummond, 2010; Kubatko et al., 2009; Liu and Pearl, 2007). Groups that contain many described but weakly differentiated (and potentially interbreeding) species, and

those that exhibit relatively little phylogenetic structure can be particularly problematic. In some cases, the most desirable solution would be an integrated approach that resolves both the species boundaries and the species phylogeny, but these methods are parameter-rich and require relatively informative input data for reliable inferences. Thus the joint resolution of phylogeny and species boundaries might not be feasible for recently diverged rapid radiations or other groups characterized by extremely low levels of genetic variation (Carstens and Dewey, 2010; O'Meara, 2010; Polihronakis, 2010; Weisrock et al., 2012).

Although clear solutions to these taxonomic and phylogenetic challenges need further development, one way forward is to hypothesize that currently-recognized species are real and then use single or multiple-exemplar sampling to estimate species trees. A critical question when following this approach is, what is the relationship between the accuracy of the hypothesized species lineages and the resulting species tree? Although seldom made explicit, two underlying assumptions often characterize this approach in the phylogenetics literature. First, the reconstruction of single-exemplar species phylogenies implies that the contained species are distinct lineages, although this is seldom tested. Second, if the named species are not distinct lineages, then both single and multiple-exemplar phylogenies should return poorly resolved trees, owing to the shifting and uncertain placement of problematic taxa or individuals within the final collection of trees. To our knowledge this relationship between correctly delimited species and resulting species trees has never been formally explored with simulated or real data. If these assumptions are correct, and taxonomic inflation (Isaac et al., 2004) or oversplitting (Dayrat, 2005) has led to the naming of indistinct lineages, then we can make two predictions about resulting species trees. It seems reasonably clear that analyses based on multiple exemplars per species should recover poorly resolved, paraphyletic species lineages. More controversially, single-exemplar trees should have low bootstrap or posterior probabilities for nodes involving these lineages. The converse logic should also hold: if phylogenies generated from single or multiple exemplars per species are well resolved, it implies that the taxa under study are themselves well-resolved lineages. One goal of the current study is to test these predictions empirically.

Turtles present many examples of taxonomically problematic groups that exhibit hybridization and introgression, incomplete coalescence, and low information content resulting from an overall reduced rate of molecular evolution in the group (Shaffer et al., 2013), ms). Taken together, these problems have produced several taxonomic controversies throughout the turtle tree of life. Well-known examples include the Mediterranean Spur-thighed Tortoise (*Testudo graeca*) complex, Australian and New Guinean members of the genus *Emydura* and the North American cooters in the genus *Pseudemys*. Each of these radiations is widespread, morphologically variable, generally common, and well studied. Each also contains one or more taxa listed as an endangered species, making it even more critical that species are accurately delimited. The *T. graeca* complex is considered to be a single, or as many as 10 species (Parham et al., 2006; Turkozan et al., 2010), while *Emydura* has been considered to comprise four species or up to seven species including eight contained subspecies (Georges and Thomson, 2010). However, within chelonians, *Pseudemys* may be the most extreme example of a taxonomically confusing group. *Pseudemys* is a group of freshwater turtles (family Emydidae, subfamily Deirochelyinae) distributed throughout the southeastern US/northern Mexico, from New Mexico, Texas and adjacent Mexico east to Florida and north to Massachusetts (Conant and Collins, 1991, Fig. 1). For turtles, which comprise only 331 living species (TTWG, 2012), *Pseudemys* is a relatively large group (7–9 recognized species), but species boundaries among most named entities are uncertain and have been the subject of extensive and

conflicting revision (Carr, 1952; Carr and Crenshaw, 1957; Fahey, 1980; Jackson, 1995; Seidel, 1994; TTWG, 2012; Ward and Jackson, 2008).

Pseudemys has long been recognized as something of a taxonomic quagmire, and has been the subject of a confusing litany of taxonomic arrangements (Fahey, 1980). For example, Leary et al. (2008, pp. 019.1) wrote:

The Alabama red-bellied turtle (*P. alabamensis*) was considered to be an invalid taxon and was designated as a "mutant of *P. floridana mobilensis*" (=*P. concinna mobilensis*) (Carr, 1938), or a variant of "*P. floridana suwanniensis*" (=*P. c. suwanniensis*) (Carr, 1952). It was also included within what is now *P. nelsoni* (De Sola, 1935), or considered a subspecies of *P. rubriventris* (Steneger, 1938; Wermuth and Mertens, 1961, 1977).

The taxonomic history of other *Pseudemys* species is similarly convoluted (Carr, 1952; Fahey, 1980; Jackson, 1995; Seidel, 1994; TTWG, 2012; Ward and Jackson, 2008), suggesting that *Pseudemys* constitutes a reasonable case study to explore the relationship between species delimitation and species-tree reconstruction in groups where both have been difficult to determine.

In this paper, we use a large, multilocus dataset to analyze both species delimitation and phylogenetic relationships across *Pseudemys*. Clarification of species boundaries and phylogeny of this clade is important for at least three reasons: *Pseudemys* constitutes an abundant part of the aquatic vertebrate fauna of the southeastern US; the currently recognized taxonomy makes *Pseudemys* a major contributor (nine taxa) to the identification of the Gulf Coast region of the US as the area of greatest chelonian species richness on earth (Buhlmann et al., 2009); and, it contains an endangered species, *P. alabamensis*, under the US Endangered Species Act (ESA). As an initial working hypothesis, we follow the TTWG (2012) and recognize nine taxa within *Pseudemys* including seven species of which one has three subspecies. As detailed in the annotations to earlier versions of the Turtle Taxonomy Working Group, both the taxonomy and content of several species complexes has remained controversial, rendering it difficult to identify a single taxonomy for *Pseudemys*. Many recent authors recognize three species, *P. alabamensis*, *P. nelsoni*, *P. rubriventris* that are assigned to the redbelly, or "*rubriventris*" group. The remaining six generally recognized taxa include *P. gorzugi*, *P. peninsularis*, *P. texana*, *P. concinna concinna*, *P. concinna floridana*, and *P. concinna suwanniensis*, which are collectively assigned to the river cooter, or "*concinna*" group (Seidel, 1994). Most current authors agree on the recognition of *P. gorzugi*, *P. texana*, and *P. concinna* as taxa; the most active debate currently centers on *P. peninsularis* (distinct species vs. subspecies of *P. floridana*), *P. floridana* (distinct species or subspecies of *P. concinna*), and *P. suwanniensis* (distinct species or subspecies of *P. concinna*). Although each of these species was initially recognized based on color pattern and morphological features, many of these characters show marked overlap among different hypothesized species (Carr, 1952; Carr and Crenshaw, 1957; Fahey, 1980; Seidel, 1994), leading to the unsettled taxonomy for the group. We follow the configuration of seven species and three additional subspecies of *P. concinna* (*concinna*, *floridana*, and *suwanniensis*) in this study.

Despite the many taxonomic revisions of *Pseudemys* (Carr and Crenshaw, 1957; Jackson, 1995; Seidel, 1994), only six phylogenetic analyses have been completed for the genus, and only two have incorporated multiple individuals/species. Stephens and Wiens (2003, 2008, 2009), and Wiens et al. (2010) generated single-exemplar species phylogenies from mitochondrial DNA (mtDNA), nuclear DNA (nuDNA) and morphological characters for the turtle family Emydidae (which includes *Pseudemys*), while Jackson et al. (2012) and Seidel (1994) focused on *Pseudemys*, generating phylogenies from multiple individuals/species (mtDNA, and morphometric plus one protein electrophoretic character,
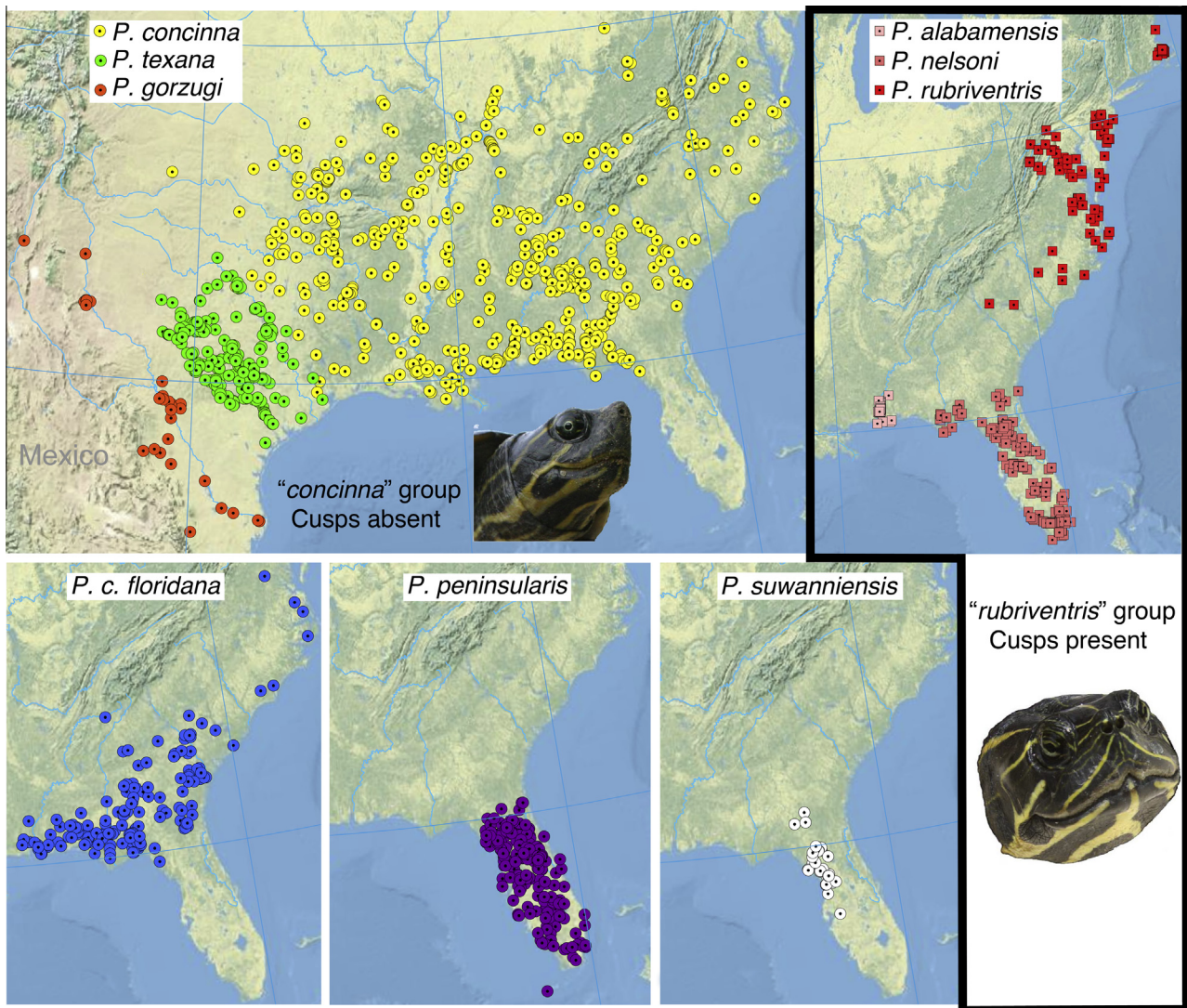
Fig. 1. Map of the eastern United States showing major rivers and known collection records as indexed in the World Turtle Database http://emys.geo.orst.edu/. Symbol colors correspond to *Pseudemys* species and subspecies (*P. c. concinna, P. c. floridana* and *P. c. suwanniensis*) as indicated. Some authors consider *floridana* and *suwanniensis* to be full species. Also shown are examples of the pair of tooth-like cusps on the upper beak, a feature that often characterizes members of "*rubriventris*" group turtles. All members of the "*concinna*" group lack these cusps except in *P. texana*, for which they may be present or absent. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

respectively). The mtDNA phylogeny of *Pseudemys* is largely unresolved (Jackson et al., 2012; Stephens and Wiens, 2003), but by using multiple individuals/species, Jackson et al. (2012) recovered *P. texana* (six individuals) and *P. gorzugi* (seven) each as monophyletic. On the other hand, the single-exemplar phylogenies of *Pseudemys* generated by Stephens and Wiens (2008, 2009) and Wiens et al. (2010) were well supported, and very similar. The analyses of Seidel (1994), Stephens and Wiens (2008, 2009), and Wiens et al. (2010) recovered *P. gorzugi* as the sister taxon to *P. texana*, and *P. nelsoni* as the sister taxon to *P. rubriventris*, in contrast to the preferred tree reported by Stephens and Wiens (2003) where *Pseudemys* formed a poorly supported pectinate subtree within the Emydidae.

Here, we employ both phylogenetic and population genetic analyses in conjunction with an 11-locus nucleotide sequence data set generated from a sparse, but range-wide sampling of multiple individuals/species to assess species boundaries and to generate a multilocus phylogeny for *Pseudemys*. We approach this problem from three directions. First, under the hypothesis that the described *Pseudemys* species are recognizable

metapopulation lineages (sensu de Queiroz, 1998, 1999), we performed Bayesian phylogenetic analyses on single and concatenated loci for the group, and assessed species boundaries using Bayesian concordance analyses. Second, recognizing that there might be little genetic differentiation within and among described species, we used Bayesian population genetic methods to assess population subdivision among putative *Pseudemys* species. These analyses indicate that several *Pseudemys* lineages are poorly differentiated, which may be the result of oversplitting. By contrast, previous single-exemplar analyses indicated well-resolved trees among these same taxa (Stephens and Wiens, 2003, 2008, 2009; Wiens et al., 2010). To explore these seemingly contradictory results more fully, we stochastically generated single exemplar phylogenies by sub-sampling our data set to assess the relationship between nodal support values and confidence of species delimitation for *Pseudemys*. Using these complementary approaches, our goals were to clarify the phylogeny of and species boundaries within *Pseudemys*, and, more generally, to consider some approaches for working with similarly challenging systematics problems.

2. Materials and methods

2.1. Taxon and data sampling

Our taxon sampling consisted of 86 individuals including four outgroups, and at least three individuals of each putative *Pseudemys* species and subspecies (Appendix 1). We concentrated our taxon sampling on *P. concinna* ssp. (56 total) for two reasons. First, much of the taxonomic confusion surrounding *Pseudemys* involves *P. concinna* and its associated (sub)species. Therefore, we included 25 *P. c. concinna*, as well as 11 *P. c. floridana*, and seven *P. c. suwanniensis* because these latter two taxa have been recognized as distinct species by some workers (e.g. Fritz et al., 2012, and Ernst and Lovich, 2009, respectively). In addition, we included 13 individuals for which no voucher existed, and that were field collected from the geographic region where *P. c. concinna* or *P. c. floridana* may occur; these individuals are labeled as *P. c. concinna/floridana* hereafter. Second, *P. concinna* and associated subspecies are widely distributed across the central and southeastern US, and our increased taxon sampling should allow us to identify geographic variation within *P. concinna*. Our outgroup sampling consisted of four emydid species including two species each of the subfamilies Deirochelyinae (*Chrysemys picta*, *Graptemys barbouri*), and Emydinae (*Emys [Actinemys] marmorata*, *Terrapene carolina*).

Our data set contains sequences for 86 individuals from up to 10 nuclear loci and three mitochondrial genes (Table 1). DNA was extracted from blood or soft tissue samples using a salt extraction protocol (Sambrook and Russell, 2001). Partial sequences of all loci were generated using 20 μl volume PCR reactions, with an initial denaturation of 60 s at 95 °C, followed by 40 cycles of denaturation (94 °C for 30 s), annealing (45 s at 60–65 °C), and extension (72 °C for 60–90 s) with a final extension period (72 °C for 10 min) (see Table 1 for locus-specific annealing temperatures, extension times and primers). All PCR products were sequenced by Beckman Coulter Genomics (http://www.beckmangenomics.com/).

2.2. Phylogenetic analyses and estimates of species trees

The mtDNA data were treated as a single locus partitioned by gene region (ND4, COI, DLOOP) and by codon for ND4 and COI, while the nuDNA markers were analyzed as 10 single loci and as a concatenated data set. Coding regions (COI, ND4 and the nuclear

exon, see Table 1) were translated using Geneious v5.1 (Drummond et al., 2010) to check for pseudogenes. Models of molecular evolution were estimated using MrModeltest 2.1 (http://www.ebc.uu.se/systzoo/staff/nylander.html) executed in PAUP*4.0b10 (Swofford, 2002). For the single-gene nuclear analyses, *E. marmorata* was identified as the outgroup allowing us to test the monophyly of *Pseudemys* with respect to *G. barbouri* and *C. picta*. We performed Bayesian phylogenetic analyses using MrBayes V3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Bayesian analyses consisted of two independent runs each comprising four incrementally heated chains that ran for 5,000,000 generations. We sampled the posterior distribution every 1000 generations, and checked for stationarity by ensuring that the average standard deviation of split frequencies between independent runs approached 0 and the potential scale reduction factor equaled 1. We examined the MCMC samples in Tracer (Rambaut and Drummond, 2009) to ensure that all chains were sampling from the same target distribution, and we discarded the first 25% of samples as burnin provided the chains had reached stationarity prior to this point.

We performed several additional analyses to assess the impact of including multiple individuals/species on the nuDNA phylogeny, as well as to assess species designations for *P. c. floridana* and *P. c. suwanniensis*. First, we selected (at random) one individual of each monotypic *Pseudemys* species as well as one individual *P. c. floridana*, *P. c. suwanniensis*, and *P. c. concinna* from the concatenated 86-individual, 6570 bp nuDNA data set and assembled these nine taxa plus the four outgroups into an alignment. For these analyses, the *P. c. concinna/floridana* individuals plus *P. peninsularis* FTA821 were excluded due to ambiguous species assignment. Next, we performed Bayesian phylogenetic analysis on each alignment with the data partitioned by locus, and then repeated this procedure an additional 99 times, generating 100 13-taxon phylogenies. MrBayes (and most other Bayesian phylogenetic-analysis software) only sample resolved trees, potentially yielding arbitrarily resolved nodes with artificially high posterior support, the "star-tree paradox" (Lewis et al., 2005; Suzuki et al., 2002; Yang, 2007). Therefore, we also analyzed all 100 13-taxon datasets under the polytomy prior implemented in Phycas (Lewis et al., 2009). For these analyses, the data were partitioned by locus, and we employed default priors with 100,000 MCMC cycles sampled every 100 cycles. We then compared the 100 MrBayes phylogenies against the 100 phylogenies generated using Phycas using symmetric tree distances (Robinson and Foulds, 1981). We also compared the 100 MrBayes

Table 1
The 10 nuclear loci and three mitochondrial genes sequenced for this analysis including marker names and PCR conditions as well as models of molecular evolution. 1st, 2nd, and 3rd refer to codon position.

Marker	Locus	Model	Primers	Temp/time (s)	Primer source
COI	mtDNA	HKY + I	L-turtCOI, H-turtCOI		Stuart and Parham (2004)
COI 1st	mtDNA	GTR + G			
COI 2nd	mtDNA	F81			
COI 3rd	mtDNA	SYM + I			
DLOOP	mtDNA	HKY + I	DES1, DES2		Starkey et al. (2003)
ND4	mtDNA	GTR + I	L-ND4, H-leu		Stuart and Parham (2004)
ND4 1st	mtDNA	HKY			
ND4 2nd	mtDNA	GTR + I			
ND4 3rd	mtDNA	HKY + I			
BMP2	Exon	HKY	BMP2 f6, r2	60/60	Townsend et al. (2008)
HMGB2	Intron	HKY	NB17483_fmod, R2	61/60	Bäckstrom et al. (2008); Barley et al. (2010),
HNFAL	Intron	HKY	HNFAL F, R	65/60	Primmer et al. (2002)
NB22519	Intron	HKY	NB22519 F, R	60/60	Bäckstrom et al. (2008)
P26S4	Intron	HKY + G	NB17367 F, R	62/60	Bäckstrom et al. (2008)
TB01	Anonymous	HKY	TB01 F, R	61/60	Thomson et al. (2008)
TB49	Anonymous	HKY	TB49 F, R	61/60	Thomson et al. (2008)
TB73	Anonymous	HKY	TB73 F, R	61/60	Thomson et al. (2008)
TB82	Anonymous	JC	TB82 F, R	61/60	Thomson et al. (2008)
TB86	Anonymous	JC	TB86 F, R	61/60	Thomson et al. (2008)

trees against one another, and the 100 Phycas trees against one another to assess the overall consistency of tree topology when using single exemplars from closely related species. The symmetric distance is the count of the number of bipartitions present in one unrooted tree but not in the other, summed across both trees; thus, the symmetric distance between two identical unrooted trees with N tip taxa is 0, and the maximum distance between two trees is $2(N - 3)$. The data-set randomization and assembly were performed in R (www.r-project.org) and the symmetric tree-distance calculations were performed using the treedist module of the Phylip 3.66 software package (Felsenstein, 2004). Finally, using the 13-taxon data sets/phylogenies, we tested each single-exemplar tree for incongruence with previous phylogenetic hypotheses using Bayesian tests of monophyly as outlined by Linnen and Farrell (2007). In a Bayesian analysis, the posterior probabilities of trees can be interpreted as the probability that those trees are correct (assuming correct model specification) (Huelsenbeck and Rannala, 2004; Linnen and Farrell, 2007). Thus, if less than 5% of the trees were retained after filtering with a given constraint tree, the null hypothesis of species-group monophyly or topological equivalency for each constraint and data partition was rejected (Buschbom and Barker, 2006; Miller et al., 2002; Linnen and Farrell, 2007). The post-burnin trees from each 13-taxon analysis were filtered against constraint trees corresponding to the hypotheses of Seidel (1994), Stephens and Wiens (2003, 2009), Wiens et al. (2010), and the *rubriventris* group vs. *concinna* group split (Fig. S1, Supporting information). The fraction of the posterior distribution of trees from each of these 13-taxon MrBayes analyses that are congruent with each hypothesis is the probability that the competing hypotheses are congruent. Thus we would fail to reject the hypothesis of incongruence at the $P = 0.05$ level if the filters retained $\geqslant 5\%$ of the trees (Buschbom and Barker, 2006; Linnen and Farrell, 2007; Miller et al., 2002). Tree filtering was performed in PAUP*, and the outgroup taxa were pruned from the phylogenies for these comparisons. In addition, in order to be most conservative, we pruned *P. alabamensis*, *P. c. floridana* and *P. c. suwanniensis* from both the constraint trees (Fig. S1) and our empirical trees, because one or more of these taxa were not included in previous phylogenetic analyses.

2.3. Bayesian concordance analysis

When confronted with incongruent gene trees, one way to summarize the phylogenetic signal from multiple gene trees is to estimate the primary concordance tree (Ané et al., 2007; Baum, 2007). The primary concordance tree contains those clades common to more than half of the individual gene trees (Baum, 2007). The concordance factor (CF) of a clade is the proportion of genes in the sample whose true tree contains that clade (the sample-wide CF), and the proportion of genes in the genome for which a clade is in the true tree is the genome-wide CF (Ané et al., 2007; Baum, 2007). Ané et al., 2007) developed a 2-stage Bayesian approach to estimate the sample-wide, and genome-wide CF. In the first stage, gene trees are estimated using standard Bayesian phylogenetic methods. In the second stage, a second MCMC procedure is used to estimate a posterior distribution of gene-to-tree maps (GTMs) using the marginal posterior distribution of gene trees from stage one as input. The sample-wide and genome-wide CFs are then estimated from the posterior distribution of GTMs. We performed Bayesian concordance analyses (BCA) using the BUCKy software (Ané et al., 2007) on the 86-taxon, 10-locus nuDNA data set with default settings except that we increased the number of chains to four (default = 1), and ran five independent analyses using different starting seeds for each analysis. In addition, the large number of unique trees in the posterior distribution of trees from the single-gene analyses made the BCA excessively computationally intensive; therefore, we included only the 95% highest

posterior density (HPD) of trees in the BCA. Finally, eight of the 82 ingroup individuals lack sequence data for at least one locus, leading to some single-locus trees with missing terminals. The Bucky software accepts trees with equal number of terminals only; thus, these eight individuals were excluded from the BCA and do not appear in the primary concordance trees.

2.4. Population assignment of individuals

Until this point, all analyses were based on the hypothesis that there are up to nine recognizable lineages within *Pseudemys*. However, the actual genetic diversity within the group might be much lower, and may not correspond closely with the current morphology-based species delimitations. To reassess population subdivision within *Pseudemys*, we performed population assignment analysis using the STRUCTURE v2.2.3 (Pritchard et al., 2000) and Structurama2 (Huelsenbeck et al., 2011) software. These methods utilize allelic data, so we used the Bayesian approach implemented in Phase2.1.1 (Stephens et al., 2001; Stephens and Donnelly, 2003) to reconstruct probable pairs of haplotypes for each nuclear locus, and used those as our input data. We used the default settings except that we accepted those haplotypes with Bayesian posterior probabilities (PP) of $\geqslant 95\%$ only; characters with $\leqslant 94\%$ PP were recoded as ambiguous data for these analyses. Both STRUCTURE and Structurama2 implement Bayesian clustering methods that allow one to estimate the number of groups (K) represented within the data by assigning individuals to populations so as to maximize Hardy–Weinberg equilibrium within populations (Pritchard et al., 2000). One major difference between the two is that, in STRUCTURE, K is fixed by the user before each run, while in Structurama2, K can be set by the user or can be treated as a random variable following a Dirichlet process prior (Huelsenbeck and Andolfatto, 2007; Huelsenbeck et al., 2011; Pella and Masuda, 2006).

Using STRUCTURE, we performed 10 replicate analyses with K set at incremental values from 1 to 10 (100,000 MCMC iterations/replicate) using the correlated allele frequency and the admixture ancestry models because they appear to perform better than the alternatives in the face of low genetic variation (Falush et al., 2003). Determining biologically meaningful values of K is obviously critical but can be challenging (Evanno et al., 2005; Pritchard et al., 2000), and the model implemented in STRUCTURE has recently been criticized as unreliable in that it recovers fewer clusters than are actually present in the data when too low a value of K is chosen (Kalinowski, 2011). We determined the optimal value of K using two methods: the well-known ΔK procedure outlined in Evanno et al. (2005) and the ΔFst method described in Campana et al. (2011), where ΔFst is analogous to ΔK, but is derived from Fst estimates rather than the $\ln P(D)$ values. Both of these analyses were performed using the CorrSieve R software package (Campana et al., 2011). We further assessed population subdivision by performing additional analyses in STRUCTURE on each of the three major groups identified in the initial STRUCTURE analysis (see below). For these analyses, we included the admixed individuals and assigned them to a nuclear group based on their q value. For example, an individual assigned to group 1 with a genotype proportion of $\geqslant 0.501$ in the initial Structure analysis was included in group 1 for the secondary analyses. No individuals were admixed at 0.50 (Appendix 1). For the Structurama2 analyses, we used the admixture model, but with K set as a random variable. These analyses were replicated 10 times using 100,000 iterations/replicate. Admixed individuals inherit some fraction of their genome (q) from each parental population (Pritchard et al., 2000), and we considered individuals with q values between 0.10 and 0.90 to be admixed (Vähä and Primmer, 2005).

## 3. Results

3.1. mtDNA phylogeny

Our mtDNA data set was composed of up to 2209 base pairs (bp) for 86 individuals (82 *Pseudemys*, four outgroups). The matrix was almost complete with ~1.6% missing data (Treebase accession # M16158), and all sequences generated here were submitted to GenBank (Appendix 1, Supporting information). The majority-rule consensus of the posterior distribution of trees from the Bayesian analysis was largely unresolved. Even with our relatively restricted sampling, only two currently recognized species, *P. gorzugi*, and *P. rubriventris*, were demonstrably monophyletic, while the clade containing all *P. alabamensis* also included a single heterospecific *P. c. floridana* (Fig. 2). However, this *P. c. floridana* is from the Magnolia River, Baldwin Co., AL, which is a known *P. alabamensis* locality and the collection site of *P. alabamensis* M12; thus, if this specimen was misidentified, *P. alabamensis* would also be recovered as monophyletic. A poorly supported clade consisting of two well-supported subclades, (one with three *P. gorzugi* samples, and the other with two samples of *P. c. concinna* and one *P. c. concinna/floridana*, all from Atlantic Flowing drainages in Georgia) were recovered as the sister clade to the remaining *Pseudemys*, while *Pseudemys rubriventris* was the sister group to a more inclusive clade containing samples assigned to *P. alabamensis*, *P. c. concinna*, *P. c. floridana*, *P. c. concinna/floridana*, *P. nelsoni*, *P. peninsularis* and *P. texana* (Fig. 2). Our result is similar to that of Jackson et al. (2012) in that *P. gorzugi* (along with two *P. c. concinna*, and a *P. c. concinna/floridana*) was the sister taxon to the remaining *Pseudemys*. However, our results differ in that Jackson et al. (2012) did not recover their three samples of *P. rubriventris* forming a monophyletic group, but did recover six *P. texana* as forming a monophyletic group. Overall, there was surprisingly little structure to the tree, with modest or weak support for most nodes.

3.2. 86-Taxon single loci and concatenated nuDNA phylogenies

Our nuDNA data set is composed of up to 6570 bp of sequence data generated from 10 loci for 86 turtles (82 *Pseudemys* plus four outgroups, TreeBase accession# M16158), and all sequences generated here were uploaded to GenBank (see Appendix 1, Supporting information for GenBank accession numbers). Sequencing chromatograms for several individuals and introns (most notably TB73) displayed patterns indicative of heterozygous length polymorphisms (see Bhangale et al., 2005), and we used the Indelligent v.1.2 software (Dmitriev and Rakitov, 2008, available at http://cta-p.inhs.uiuc.edu/dmitriev/indel.asp) to reconstruct nucleotide sequences from chromatograms disrupted by heterozygous length polymorphisms. Analyses of six individual loci, and the 10-locus concatenated data set failed to converge using the models selected through MrModelTest as determined by examination of trace files from the posterior distributions. Failure to converge can be due to model overparameterization (Rannala, 2002), so we simplified the models for these data sets and reran the analyses (final models shown in Table 1). The single-locus analyses all appeared to converge using these simplified models, but the concatenated analysis still failed to converge. Convergence was reached only after running this analysis for an additional 45,000,000 MCMC steps. The phylogeny from this analysis was mostly unresolved, but there was support for monophyly of *P. gorzugi* and a grouping of the two Texas endemic taxa *P. gorzugi* and *P. texana*, with *P. texana* recovered as paraphyletic with respect to *P. gorzugi*. Also, our six *P. peninsularis* samples plus a *P. c. floridana* (FLMNH UF159336) were recovered as a clade (Fig. 3).
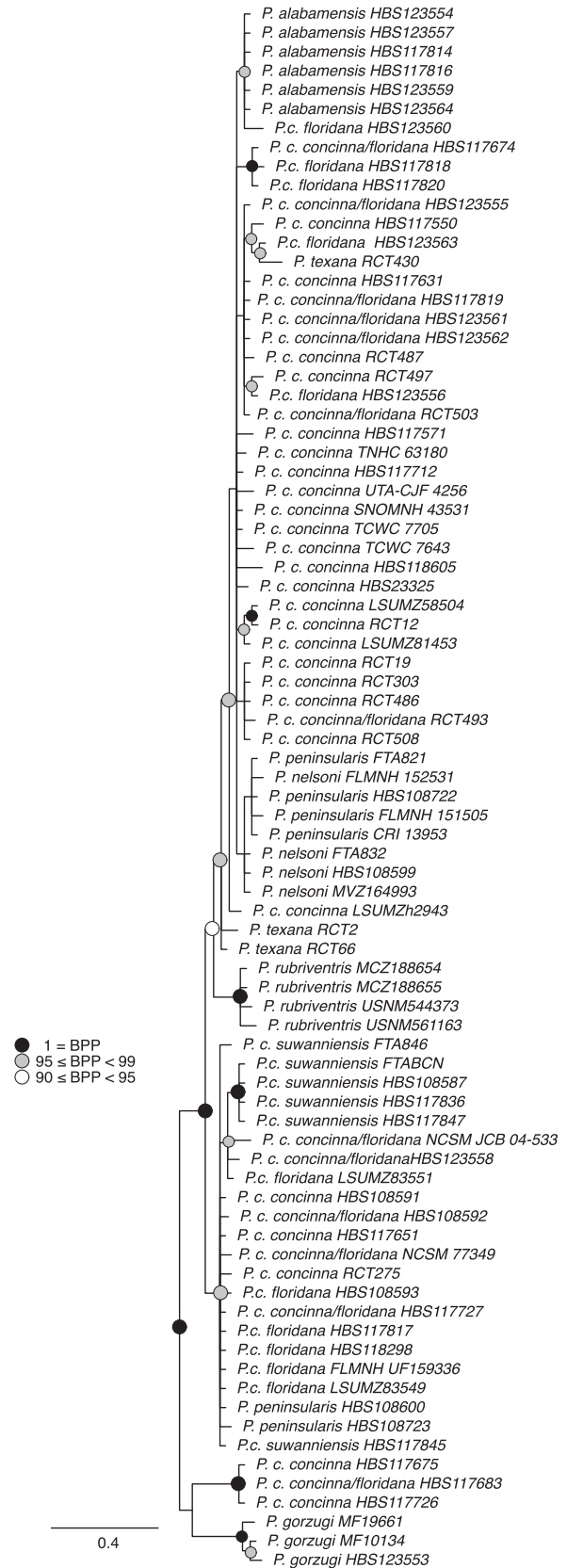
Fig. 2. Majority-rule consensus of the posterior distribution of trees from the Bayesian analysis of the concatenated *COI*, *DLOOP* and *ND4* data set (86 individuals, 2209 bp). Terminals labeled "*P. c. concinna/floridana*" are individuals that are morphologically intermediate between *P. c. concinna* and *P. c. floridana* and could not be confidently assigned to either subspecies. Bayesian posterior probabilities (PP) as indicated. All but one outgroup was removed for clarity of presentation.
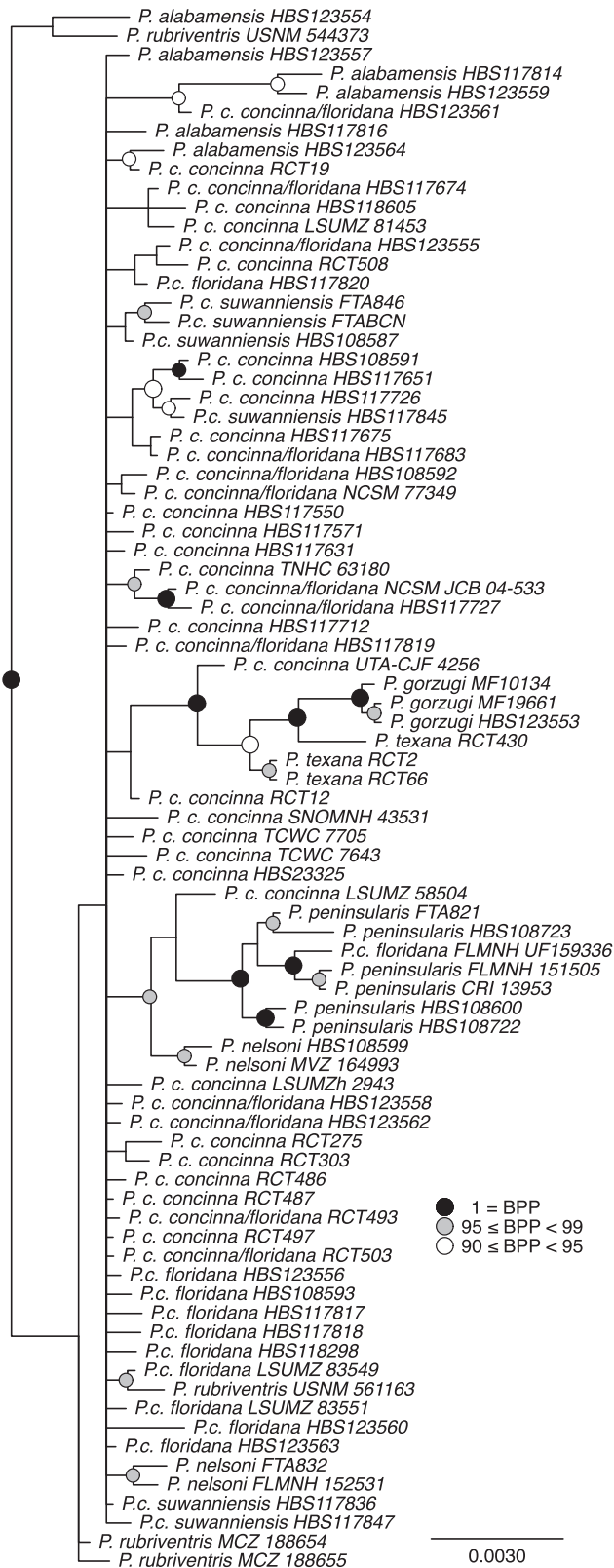
Fig. 3. Majority rule consensus tree of the posterior distribution of trees from the Bayesian analyses of the concatenated nuclear loci data (10 loci, 6570 bp). Terminals labeled "*P. c. concinna/floridana*" are individuals that are morphologically intermediate between *P. c. concinna* and *P. c. floridana* and/or could not be confidently assigned to either subspecies. Models of molecular evolution for each locus are shown in Table 1. Bayesian support values (PP) as indicated, and all but one outgroup were removed for clarity of presentation.

The gene trees from individual loci were largely unresolved (Figs. S2–S6, Supporting information), and we failed even to recover a monophyletic *Pseudemys* for five loci (Figs. S2B, S4A, B, S5B, and S6A). However, the grouping of *P. gorzugi* and *P. texana* was evident at the HNFL, NB22519, and TB86 loci (Figs. S3A, S3B, and S6B), while *P. gorzugi* was recovered as monophyletic at the BMP2 and TB49 loci (Figs. S2A and S5A). In addition, the grouping of *P. peninsularis* was evident at *BMP2* and *NB22519* (Figs. S2A and S3B, respectively).

The almost complete lack of resolution for our single-gene trees was somewhat surprising, and might be due to very low levels of genetic variation. To assess the relative genetic variability among *Pseudemys* for the nuclear loci used here, we generated pairwise uncorrected "P" genetic distances for all 100 13-taxon alignments (with outgroups excluded), and averaged these distances across all 100 alignments. To place these distances in a relevant context, we also generated, for the same loci, uncorrected "P" distances among all contained species for two genera of the turtle family Geoemydidae; *Cuora,* which contains 12 species and *Heosemys*, which contains four. We generated pairwise sequence divergence within *Cuora* and *Heosemys* in a similar manner as for *Pseudemys*. Here we selected single exemplars/species with replacement from concatenated nuDNA sequence alignments of *Heosemys* and *Cuora* reported in Spinks et al. (2012a) and Spinks et al. (2012b), respectively. We then assembled the single exemplars into alignments, generated interspecific pairwise uncorrected "P" genetic distances, and repeated this process an additional 99 times. The results are shown in Table 2. Based on these comparisons, the mean interspecific sequence divergence among *Pseudemys* was less than that of *Cuora* and *Heosemys* at all assayed loci (7 loci and 5 loci, respectively), but at two loci, the maximum observed sequence divergence was higher among *Pseudemys* than either *Cuora* or *Heosemys* (Table 2).

In summary, the mtDNA provided some support for the monophyly of *P. gorzugi*, *P. rubriventris* and *P. alabamensis*, while the concatenated and single-locus nuclear gene trees indicated that *P. gorzugi* and perhaps *P. peninsularis* were monophyletic, and that the two Texas endemics, *P. gorzugi* and *P. texana* form a clade. Little strong support was found for other traditionally recognized groups, either for individual species or the more inclusive *concinna* or *rubriventris* groups (Table 3).

3.3. 13-Taxon single exemplar concatenated nuDNA phylogenies

The trees generated from analyses of the 13-taxon data sets were generally well supported but highly incongruent. Each data set contained nine terminals (we excluded the outgroups for these comparisons), and therefore up to eight internal nodes. Among all replicates, 79/100 trees were fully resolved using MrBayes, but only 19/100 using Phycas (Figs. S7 and S8, Appendix 2). The average tree was very well supported, particularly in the MrBayes analyses, where on average 6/8 internal nodes were supported by Bayesian posterior probability [PP] values >95 and 21/100 trees had PP > 95 at all nodes. Under Phycas, support was somewhat weaker, with ~5/8 nodes supported by PP values >95, and 3/100 trees supported by PP > 95 at all nodes (Figs. S7 and S8, Appendix 2, Supporting information). The average symmetric distance among all pairwise comparisons was ~8 in the MrBayes analyses and ~6.5 in the Phycas analyses, and the average symmetric distance between trees generated using MrBayes vs. those generated using Phycas was 1.5 (Appendix 2). In the Bayesian tests of monophyly using trees generated from the MrBayes analyses, 97/100 trees were incongruent with Seidel (1994), 97/100 were incongruent with Stephens and Wiens (2003), 79/100 were incongruent with Stephens and Wiens (2009), and 76/100 were incongruent

Table 2

Comparisons of mean (top) and maximum (bottom) intrageneric pairwise uncorrected "P" genetic distances among three genera of turtles for the 10 loci used in this analysis. For example, for *Pseudemys* at the BMP2 locus the mean intrageneric P distance was 0.0019 while the maximum P distance was 0.0102. NA indicates loci not included in that particular analysis.

Species	Nuclear loci									
	BMP2	HMGB2	HNFL	NB22519	P26s4	TB01	TB49	TB73	TB82	TB86
Cuora[a]	0.0035	0.0171	0.0142	0.0100	0.0091	0.0054	NA	0.0148	NA	NA
	0.0096	0.0513	0.0309	0.0227	0.0186	0.0154		0.0413		
Heosemys[b]	0.0067	0.0251	0.0152	0.0301	NA	0.0063	NA	NA	NA	NA
	0.0114	0.0410	0.0246	0.046		0.0087				
Pseudemys[c]	0.0019	0.0013	0.0078	0.0045	0.0029	0.0008	0.0058	0.0035	0.0068	0.0056
	0.0102	0.0071	0.0404	0.0166	0.0085	0.0063	0.0388	0.0143	0.0175	0.0552

[a] Values generated using data from Spinks et al. (2012b).
[b] Values generated using data from Spinks et al. (2012a).
[c] This study.

Table 3

Summary of support for *Pseudemys* species. "Yes" as an entry indicates some support for that taxon based on the molecular results in this paper, even if monophyly at the phylogenetic level is not absolute.

Species	Phylogenetic analyses			Other analyses	
	mtDNA	Single gene	Concatenated nuDNA	BCA	STRUCTURE
P. alabamensis	Yes[a]				Yes[a]
P. concinna					
P.c. floridana					
P.c. suwanniensis					
P. gorzugi	Yes	Yes	Yes	Yes	Yes
P. nelsoni				Yes	
P. peninsularis		Yes	Yes	Yes	Yes
P. rubriventris	Yes				
P. texana					

[a] Nonmonophyly of *P. alabamensis* could be due to introgression, specimen misidentification, or sample mislabeling.

with the *rubriventris* vs. *concinna* group split (Appendix 2). Finally, the average symmetric distance among the 21 MrBayes trees with all nodes supported by PP > 95 was 6.7. Thus, on average most of single-exemplar concatenated-gene trees were well supported, but were also highly incongruent with one another and with previous phylogenetic hypotheses.

3.4. Bayesian concordance analyses

The primary concordance trees from the five replicate analyses were very similar, and varied at only a few minor branches; we show a single representative tree from these analyses in Fig. 4. As in the 83-taxon concatenated nuDNA tree (Fig. 3), the primary concordance trees were mostly unresolved, and CFs were generally low across the trees. The main results across all five replicates were the monophyly of *P. gorzugi*, and the monophyly of *P. peninsularis* (Fig. 4).

3.5. Population assignment analyses

The STRUCTURE analyses indicated that 20 individuals were admixed, but 62 were assigned with high probability ($\geqslant 90\%$) into one of three groups (Appendix 1). Of these, only one group (group 3) containing five individuals was geographically cohesive (group 3 in Fig. 5) and corresponds to our *P. gorzugi* and 2/3 of the *P. texana* samples. The two remaining Texas individuals that formed a geographically cohesive clade in the concatenated nuclear results (Fig. 3) were found to be admixed (Fig. 5; Appendix 1). The remaining groups (1 and 2) overlap widely in the southeast, especially northern Florida, and the eastern Gulf Coast region. Group 1 contained 23 samples from turtles assigned to *P. c. concinna*, *P. c. floridana*, *P. c. concinna/floridana* and *P. c. suwanniensis*, while group 2

contained 34 samples from turtles assigned to *P. alabamensis*, *P. concinna*, *P. c. floridana*, *P. c. concinna/floridana*, *P. nelsoni*, *P. peninsularis*, *P. rubriventris*, and *P. texana*. Finally, the group 1 × group 2 intergrades included 18 specimens originally classified as *P. concinna*, *P. c. floridana*, *P. c. concinna/floridana*, and *P. rubriventris*, and the group 2 × group 3 intergrades (two individuals) included one individual each that was initially assigned to *P. texana* and *P. c. concinna* (Fig. 5).

When the group 1 individuals were subjected to additional analyses, ΔK values for $K = 1$–3 were very similar as was ΔFst (not shown), and both indicated that there was no additional population substructure. However, analyses of group 2 revealed some additional fine-scale population substructure. Here, we found an optimal value of $K = 4$ (identified as subgroups 2a–2d), with 24 individuals assigned to subgroup 2a (16 individuals at $\geqslant 0.90$) including turtles originally assigned to *P. c. concinna*, *P. c. floridana*, and *P. c. concinna/floridana*, as well as one individual each of *P. alabamensis* and *P. rubriventris* assigned at $\leqslant 0.90$. Interestingly, subgroup 2b contained all six *P. peninsularis*, and all were assigned with probabilities >0.95 (Appendix 1). Subgroup 2c contained six individuals including five *P. alabamensis* and one *P. rubriventris*. Finally, subgroup 2d contained seven individuals including one *P. c. concinna*, 2 *P. rubriventris* and all four *P. nelsoni* (Appendix 1). On the other hand, the Structurama2 analyses of the full 82-taxon data set where K was not fixed to a predetermined value failed to reveal conclusive evidence for population substructure, and the posterior probability for the number of groups was $\leqslant 0.07$ in all analyses. To explore these results, we ran additional analyses with the number of populations treated as a random variable, but with the prior for K set from 2 to 10. These analyses also failed to recover any significant population structure.

Fig. 4. One of five primary concordance trees generated from the 95% highest posterior density of trees from 10 nuclear loci. Concordance factors are shown above branches. Terminals labeled "*P. c. concinna/floridana*" are individuals that are morphologically intermediate between *P. c. concinna* and *P. c. floridana* and/or could not be confidently assigned to either subspecies. The outgroups were removed for clarity of presentation.

4. Discussion

Our analyses provide insights into the problematic taxonomic and phylogenetic history of *Pseudemys*, and also highlight some important cautions and pitfalls associated with performing phylogenetic analyses on complexes of closely related species.

4.1. Phylogeny and species delimitation of Pseudemys

Our analyses do not support the currently hypothesized species limits or more inclusive species groups for *Pseudemys*. Of the seven recognized species in the most current species list for chelonians (TTWG, 2012), only *P. gorzugi* was consistently recovered as monophyletic across analyses (Table 3), although *P. peninsularis* was recovered with nuclear (but not mt) DNA. Such wholesale incongruence with current taxonomy could be due to at least three non-mutually exclusive reasons: (1) methodological issues

including uninformative markers, incomplete sampling or inappropriate analyses; (2) incorrect sample identification; or (3) oversplitting of a smaller number of morphologically variable evolutionary lineages. We discuss each in turn below.

We feel reasonably confident that methodological and specimen-identification issues (possibilities 1 and 2) are not responsible for our results. Obviously, marker choice is an important consideration in molecular analyses, and the goal in any analysis is to select a set of markers that are evolving at a rate appropriate for the group/questions of the particular study. Most of the nuclear markers employed here have proven useful for intra and interspecific phylogenetic analyses of other turtle genera (Table 2, Spinks et al., 2012a, 2012b). Although we view comparisons of genetic distances, like those in Table 2, as a simple heuristic tool, our results indicate that the markers employed here are variable enough to allow for species delimitation and species-tree reconstructions (particularly for *Heosemys*, Spinks et al., 2012a) when species are well
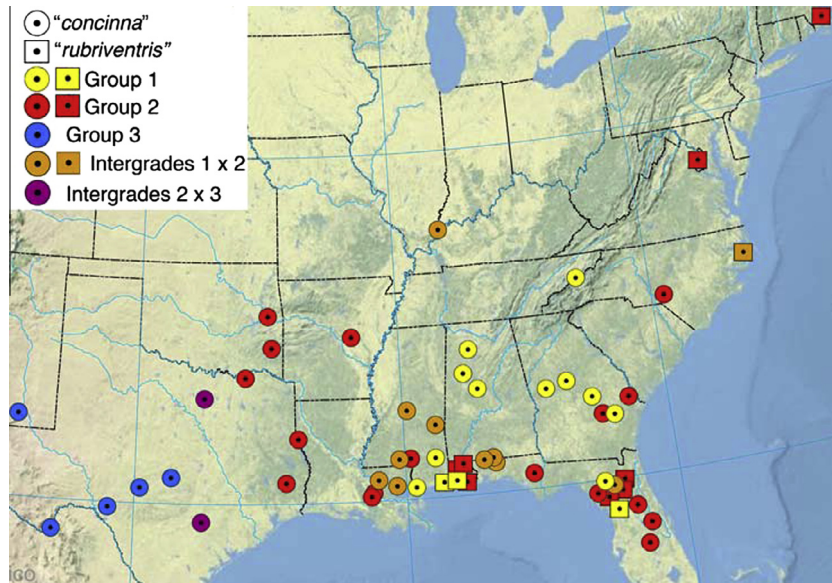
Fig. 5. Results of the STRUCTURE analyses overlain on collection locality. Circles indicate individuals assigned to the "*concinna*" group based on field/museum morphological identification while squares indicate individuals assigned to the "*rubriventris*" group. Colors indicate groupings determined from the STRUCTURE analysis: yellow = group 1 individuals, red = group 2, and blue = group 3. orange = group 1 × group 2 intergrades and purple = group 2 × group 3 intergrades. No group 1 × group 3 intergrades were identified. In some instances, multiple samples were combined into a single locality. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

demarcated. Thus, the extremely low levels of genetic differentiation that we found in *Pseudemys* are unlikely to be purely an artifact of marker selection. While most markers showed lower between-species levels in *Pseudemys* compared to *Heosemys* or *Cuora*, our concatenated single-exemplar trees contain sufficient variation to return well-supported trees, suggesting again that there is sufficient variation in our data set. Our analyses included population-genetic approaches (STRUCTURE, Structurama2) developed to uncover subtle population differentiation (Rosenberg et al., 2001) and to provide a discriminating first pass in species-delimitation studies (Shaffer and Thomson, 2007). Our sampling, while sparse for each taxon, is geographically comprehensive, and it represents a reasonable span of potential variation within nominal species.

Correctly identifying samples is also obviously critical, and some *Pseudemys* species and subspecies are notoriously difficult to identify, particularly in the Gulf Coast region where our field work and those of colleagues have identified many potential intergrades (Godwin, pers. comm., Jackson, pers. comm., Shaffer and Pauly, unpublished results). However, the bulk of our samples come from vouchered museum samples and/or were collected by experts at *Pseudemys* identification (see Acknowledgements). They are presumably identified to species as well as possible (and as well as any other specimens on which these species are based), given the current disagreements within the community. In addition, diagnosis of the *rubriventris* and *concinna* species groups is generally considered more straightforward (based on plastral coloration and the presence/absence of a pair of tooth-like cusps on the upper beak), yet this primary split of our *Pseudemys* samples into these two groups was rare in single-exemplar trees (24/100 trees, Appendix 2) and absent from multi-exemplar analyses.

The interpretation most consistent with these data is that *Pseudemys* evolutionary diversity has indeed been oversplit. One working hypothesis is that the genetic diversity among *Pseudemys* may best be represented by three lineages, given that STRUCTURE population-assignment analyses indicated that three groups with slight subpopulation structure within group 2. The correlated allele frequency model in STRUCTURE often performs well in inferring the correct number of clusters in the face of low levels of genetic

divergence (Latch et al., 2006, but see Kalinowski, 2011). This three-lineage model would include one taxon consisting of the westernmost populations of *Pseudemys* currently assigned to *P. texana* plus *P. gorzugi* (group 3 in Fig. 5), one lineage that is widespread and geographically variable, ranging from east Texas and the Mississippi drainage in the west across the Gulf Coast and peninsular Florida and north along the eastern US coastal plain to Massachusetts (group 2 in Fig. 5), and a third, somewhat less widespread lineage in the Alabama-Coosa river and nearby drainages, extending east into peninsular Florida (group 1, Fig. 5). The extent of intergradation between groups 1 and 2 as well as the northern extent of group 1 along the Atlantic Coast is not clear in our analyses because we have limited sampling in this region where *P. c. concinna*, *P. c. floridana*, and *P. rubriventris* overlap. Interestingly, our finding of extensive overlap and intergradation of groups 1 and 2 in the Gulf Coast region is consistent with previous work that revealed extensive morphological variation including, overlap of morphological characters claimed to be taxonomically important, in this area (Fahey, 1980; Mount, 1975; Seidel, 1994, 1995).

Although this three-lineage interpretation appears to be the best representation of our genetic data, we emphasize that other interpretations are possible and that morphological and biogeographic evidence should be important considerations before any future taxonomic changes are considered. For example, of the nine currently recognized taxa, at least one analysis could be construed as supporting the recognition of two members of the *rubriventris* group (*P. alabamensis* and *P. rubriventris*), and two of the *concinna* group (*P. gorzugi* and *P. peninsularis*) (Table 3). Thus, with additional samples/genes, perhaps combined with morphological analyses, might provide evidence for the other currently recognized taxa. However, no such evidence exists in our current data set, and it is the largest molecular study currently available.

Seidel (1994) argued for eight species of *Pseudemys* based on his morphometric analysis. This view, although itself controversial (e.g., Jackson, 1995) is in strong contrast with the patterns in our data. One potential explanation is that the sampling in Seidel's (1994) morphological analysis, although quite extensive, does not fully explain the extreme morphological variation observed within

what might be few actual taxa. For example, extensive morphological variation that is structured by habitat or by watershed could be easily misinterpreted as being associated with taxonomic boundaries if populations from areas intermediate in habitat or geography are not adequately sampled. Seidel and Palmer (1991) suggested just such a case in their examination of *P. c. floridana*-type turtles from the Atlantic Coastal Plain and *P. c. concinna*-type turtles from more upland areas of the Piedmont. In sampling from regions close to the Fall Line, which is the boundary between the two regions, they failed to find support for morphologically distinct taxa. Similarly, Jackson (1995) argued that the watershed- and region-specific taxa *P. c. suwanniensis* and *P. peninsularis* are not morphologically unique when one considers more detailed sampling of surrounding areas.

An alternative explanation for the apparent discrepancy between Seidel's (1994) morphological analysis and our genetic results is that Seidel's (1994) morphological analysis does indicate numerous distinct taxa, but that extensive, previously unrecognized hybridization and introgression explain our results. Formally distinguishing between these alternatives would require a joint morphological and genetic analysis (Parham et al., 2013) where both data types are gathered from an identical set of individuals, ideally using a high density of molecular markers. Thus, much more detailed sampling, including more comprehensive genomic analyses, is needed before any taxonomic changes should be implemented, and we present the current results as hypotheses requiring further testing (Pauly et al., 2009; TTWG, 2007).

4.2. Single-exemplar sampling, nodal support, and confidence in species delimitation

It is now abundantly clear that due to gene tree-species tree conflicts, phylogenetic analyses of concatenated sequence data can be misleading (Avise, 1989; Degnan and Rosenberg, 2006; Hudson and Coyne, 2002; Maddison, 1997; Maddison and Knowles, 2006; Moore, 1995; Pamilo and Nei, 1988). In addition, our results readily demonstrate that among closely related taxa, the individual selected for analysis can also dramatically impact the phylogeny (Carstens and Knowles, 2007; Shaw and Small, 2005). We recovered up to 22 fully-supported, but highly incongruent phylogenies depending on which individuals were included in the analyses (Figs. S7 and S8), while the concatenated, fully partitioned phylogeny generated from the entire data set was mostly unresolved (Fig. 3). Based on resampling of our current data set, single-exemplar trees often have uniformly strong nodal support when data are concatenated, partitioned, and analyzed. However, the resulting phylogenies may depend more on the individuals sampled than on the actual underlying species tree, calling into question the interpretation of phylogenies generated from single-exemplar sampling as accurate estimates of species trees (e.g. Stephens and Wiens, 2009; Spinks et al., 2004). Although the generality of our results requires confirmation in other systems, it appears that, at least in *Pseudemys*, strongly supported single-exemplar species trees give a misleadingly confident picture of the validity of the contained species and their phylogenetic relationships.

5. Conclusions

Based on the previous literature and the analyses presented here, delimiting species and reconstructing a species phylogeny for *Pseudemys* remain extremely daunting, but important tasks. Variability in the key morphological characters used for species delimitation is high and remains incompletely quantified (see Carr, 1952), and the modest levels of genetic variation within the genus (Table 2) may be confounding molecular species delimitation

methods (Shaffer and Thomson, 2007). Without a clear consensus on the correct assignment of individuals to taxa, some of the newest species delimitation methods, which require that individuals be assigned to species *a priori*, are virtually impossible to implement. These same models generally assume that there is no significant ongoing gene flow (i.e. *BEAST, Heled and Drummond, 2010; BEST, Liu and Pearl, 2007; and BPP, Yang and Rannala, 2010), a condition at odds with our molecular data and previous morphological interpretations (e.g. Seidel, 1994, 1995) which indicate considerable admixture, particularly in the Gulf Coast region (Fig. 5). Based on the currently accepted taxonomy, the US Gulf Coast has the highest species richness of turtles on earth (Buhlmann et al., 2009), and *Pseudemys* contains what may be the most endangered turtle on the continent (*P. alabamensis*). Species delimitation is therefore critical to our understanding of biodiversity and conservation, and requires a thorough re-evaluation of this problematic group of turtles.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2013.03.031.

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