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Genetic introgression and hybridization in Antillean freshwater turtles (*Trachemys*) revealed by coalescent analyses of mitochondrial and cloned nuclear markers

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ABSTRACT

Determining whether a conflict between gene trees and species trees represents incomplete lineage sorting (ILS) or hybridization involving native and/or invasive species has implications for reconstructing evolutionary relationships and guiding conservation decisions. Among vertebrates, turtles represent an exceptional case for exploring these issues because of the propensity for even distantly related lineages to hybridize. In this study we investigate a group of freshwater turtles (*Trachemys*) from a part of its range (the Greater Antilles) where it is purported to have undergone reticulation events from both natural and anthropogenic processes. We sequenced mtDNA for 83 samples, sequenced three nuDNA markers for 45 samples, and cloned 29 polymorphic sequences, to identify species boundaries, hybridization, and intergrade zones for Antillean *Trachemys* and nearby mainland populations. Initial coalescent analyses of phased nuclear alleles (using *BEAST) recovered a Bayesian species tree that strongly conflicted with the mtDNA phylogeny and traditional taxonomy, and appeared to be confounded by hybridization. Therefore, we undertook exploratory phylogenetic analyses of mismatched alleles from the "coestimated" gene trees (Heled and Drummond, 2010) in order to identify potential hybrid origins. The geography, morphology, and sampling context of most samples with potential introgressed alleles suggest hybridization over ILS. We identify contact zones between different species on Jamaica (*T. decussata* × *T. terrapen*), on Hispaniola (*T. decorata* × *T. stejnegeri*), and in Central America (*T. emolli* × *T. venusta*). We are unable to determine whether the distribution of *T. decussata* on Jamaica is natural or the result of prehistoric introduction by Native Americans. This uncertainty means that the conservation status of the Jamaican *T. decussata* populations and contact zone with *T. terrapen* are unresolved. Human-mediated dispersal events were more conclusively implicated for the prehistoric translocation of *T. stejnegeri* between Puerto Rico and Hispaniola, as well as the more recent genetic pollution of native species by an invasive pet turtle native to the USA (*T. scripta elegans*). Finally, we test the impact of introgressed alleles using the multispecies coalescent in a Bayesian framework and show that studies that do not phase heterozygote sequences of hybrid individuals may recover the correct species tree, but overall support for clades that include hybrid individuals may be reduced.

1. Introduction

The potential for conflicts between gene trees and species trees has been understood for almost 30 years (Tajima, 1983). Two of the main causes for conflicting trees are incomplete lineage sorting of ancestral polymorphisms (ILS) and hybridization (Edwards, 2009). Statistical methods for distinguishing ILS versus *ancient* hybridization have been proposed (Roos et al., 2011; Yu et al., 2011), but distinguishing between ILS and *recent or ongoing* hybridization is more challenging (Joly et al., 2009; Gerard et al., 2011; Spinks et al., 2012b). Identifying hybridization is key to interpreting systematic results because hybridization violates the assumptions of

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most systematic methods, including those of coalescent theory. It is also important because the identification of hybridization can inform taxonomic decisions and, in the case of genetic introgression from a non-native species, has major conservation implications (Allendorf et al., 2001; Perry et al., 2002; Fong and Chen, 2010; Ricciardi and MacIsaac, 2011; Spinks et al., 2012b).

Among vertebrates, turtles represent an exceptional case for exploring these issues because of the propensity for even distantly related lineages to hybridize (Spinks et al., 2004; Buskirk et al., 2005), combined with the fact that turtles are heavily exploited and so are brought together through human-mediated dispersal (translocations) (Parham et al., 2001; Poulakakis et al., 2008). Recent studies have revealed complex patterns of genetic variation from natural, prehistoric lineage reticulations (Shi et al., 2005; Spinks and Shaffer, 2009; Jackson et al., 2012), as well as those arising from anthropogenic causes (Parham et al., 2001; Fong et al., 2007; Fong and Chen, 2010). In this study we investigate a group of turtles (*Trachemys*) from a part of its range (the Greater Antilles) where it is purported to have undergone reticulation events from both natural and possible unnatural (anthropogenic) processes (Seidel and Inchaustegui Miranda, 1984; Tuberville et al., 2005).

Trachemys naturally co-occur with many other genera of freshwater turtles in mainland North and South America, but are the only freshwater turtle native to the West Indies. They are thought to occur naturally in the Greater Antilles (six taxa) and possibly the Bahamas (one or two taxa) (Turtle Taxonomy Working Group, 2007b). These six to eight West Indian taxa form a clade within *Trachemys* (Jackson et al., 2008; Turtle Taxonomy Working Group, 2011). The most recent taxonomic assessment of Antillean *Trachemys* relied on morphometrics and allozyme electrophoresis (Seidel and Adkins, 1987; Seidel, 1988), and so a comparative study using modern molecular methods is due. This is especially true considering that genetic study of other poorly known turtles has revealed neglected diversity (e.g., Parham et al., 2004; Fong et al., 2007; Spinks et al., 2012a), shown that morphologically defined taxa require revision (e.g., Stuart and Parham, 2004; Shi et al., 2008a), and identified human-mediated dispersal events (Poulakakis et al., 2008). These issues beg for resolution because the endemic fauna of the Greater Antilles is threatened with extinction (Hedges, 2006b; Wilson et al., 2006; Hedges and Díaz, 2011).

Although the focus of our study is on Antillean *Trachemys*, we also provide data for mainland Caribbean *Trachemys* and other North American forms as outgroups. Among the latter is *Trachemys scripta elegans* (TSE hereafter), also called the "red-eared slider" because of its prominent red postorbital stripe (a feature shared with some other *Trachemys*, including half the West Indian taxa [Fig. 1]). TSE is bred by the millions in industrial-scale turtle farms in the USA and in China (Shi et al., 2007, 2008b) and sold around the world as pets. Consequently, TSE is the most common pet-trade turtle worldwide. One consequence of this massive trade is that unwanted pets often become established as a feral invasive species. Introduced TSE are threats to the integrity of natural ecosystems as well as direct competitors and pathogen vectors for native turtles (IUCN Invasive Species Specialist Group, 2000; Platenburg, 2007). When TSE are introduced into areas where other *Trachemys* taxa occur (e.g., the Greater Antilles) there is the added threat of genetic introgression through hybridization. In addition to describing natural patterns of hybridization, identifying genetic introgression from feral TSE is one of the goals of our study.

We generated DNA sequence data from 83 samples, including 79 from the Greater Antilles and adjacent Caribbean region, to identify species boundaries, hybridization, and intergrade zones, as well as the overall genetic diversity of *Trachemys* in the region. To identify hybrids with nuDNA data, we sequenced 45 of these samples for three nuclear markers, isolated alleles by cloning, and then analyzed them in a coalescent framework. In addition to revealing the evolutionary history of these turtles, these analyses allow us to test whether the presence of phylogenetically disparate alleles in our study results from ILS or ongoing hybridization. Finally, we assess the impact of potential introgressed alleles on species tree analyses in *BEAST.

2. Materials and methods

2.1. Samples and marker selection

Our study is based entirely on known-locality genetic samples with corresponding museum voucher specimens (Appendix). For all 83 samples we sequenced a mtDNA marker ('ND4') that has been widely used for emydid turtles, including *Trachemys* (Feldman and Parham, 2002; Spinks et al., 2010; Fritz et al., 2011). Most (65) of the 83 samples sequenced for mtDNA are from the Antillean ingroup (Fig. 1). We also sequenced a subset of 45 samples (29 Antillean) for three nuclear markers (HNF-1α, RELN, R35) that have been applied to emydid turtles (Spinks and Shaffer, 2009; Spinks et al., 2009a,b). All 14 samples of adjacent mainland populations were sequenced for nuDNA to test for possible genetic contributions to Antillean populations. Our study is not aimed at resolving the relationship of non-Antillean *Trachemys*, but the extent of our outgroup sampling in Central America allows us to make some new inferences about the populations in that region. We include three samples of northern *Trachemys* in order to test for the genetic introgression from the invasive TSE, and one sample of a non-*Trachemys* deirochelyine emydid (*Pseudemys gorzugi*) for rooting purposes.

Taxonomic identification of ingroup samples was based on morphological characteristics of the voucher specimens, such as the color and amount of head striping and plastral patterning (Seidel, 1988). Subspecific identifications of outgroup *Trachemys* in the Southern Mainland clade are based on geography following published studies (Fig. 2D; Seidel, 2002; McCord et al., 2010). Six of the 65 Antillean specimens sequenced were deemed to be intergrades based on a mixture of characters. Two *T. terrapen* (samples 36 and 37) from near the newly recognized range of *T. decussata* on Jamaica showed evidence of a faint red postorbital stripe, a *T. decussata* character. Three samples from a known intergrade zone on Hispaniola (46–48) showed orange postorbital stripes (*T. decorata* have pale or yellow, whereas *T. stejnegeri* have red), sometimes combined with plastra that were partially spotted (a *T. decorata* character) and partially showing waving parallel lines (a *T. stejnegeri* character). A fourth sample (45) from deep within this area of intergradation was identified as *T. stejnegeri* based on morphology, but it shows closer affinity with *T. decorata* with all genetic markers tested here. This sample is considered an intergrade for the sake of simplicity. Finally, one sample from a TSE-infested pond on Puerto Rico showed very bold coloration evocative of TSE. All intergrades are indicated on Figs. 1 and 2.

2.2. Extractions/PCR/sequencing

Genomic DNA was extracted from approximately 25 mg of liver tissue using the Qiagen DNeasy tissue kit (Qiagen, Valencia, CA) following manufacturer's instructions. A portion of the NADH dehydrogenase subunit 4 (ND4) gene, the complete tRNAs Histidine and Serine, and part of the tRNA Leucine were amplified using primers L-ND4 and H-Leu (Stuart and Parham, 2004). In addition, three nuclear markers were amplified: intron 61 of the Reelin gene (RELN) using primers RELN61F and RELN61R (Spinks and Shaffer, 2007), intron 2 of the hepatocyte nuclear factor 1α (HNF-1α) using primers HNFAL-F and HNFAL-R (Primmer et al., 2002), and intron 1 of the fingerprint protein 35 (R35) using primers R35Ex1 and

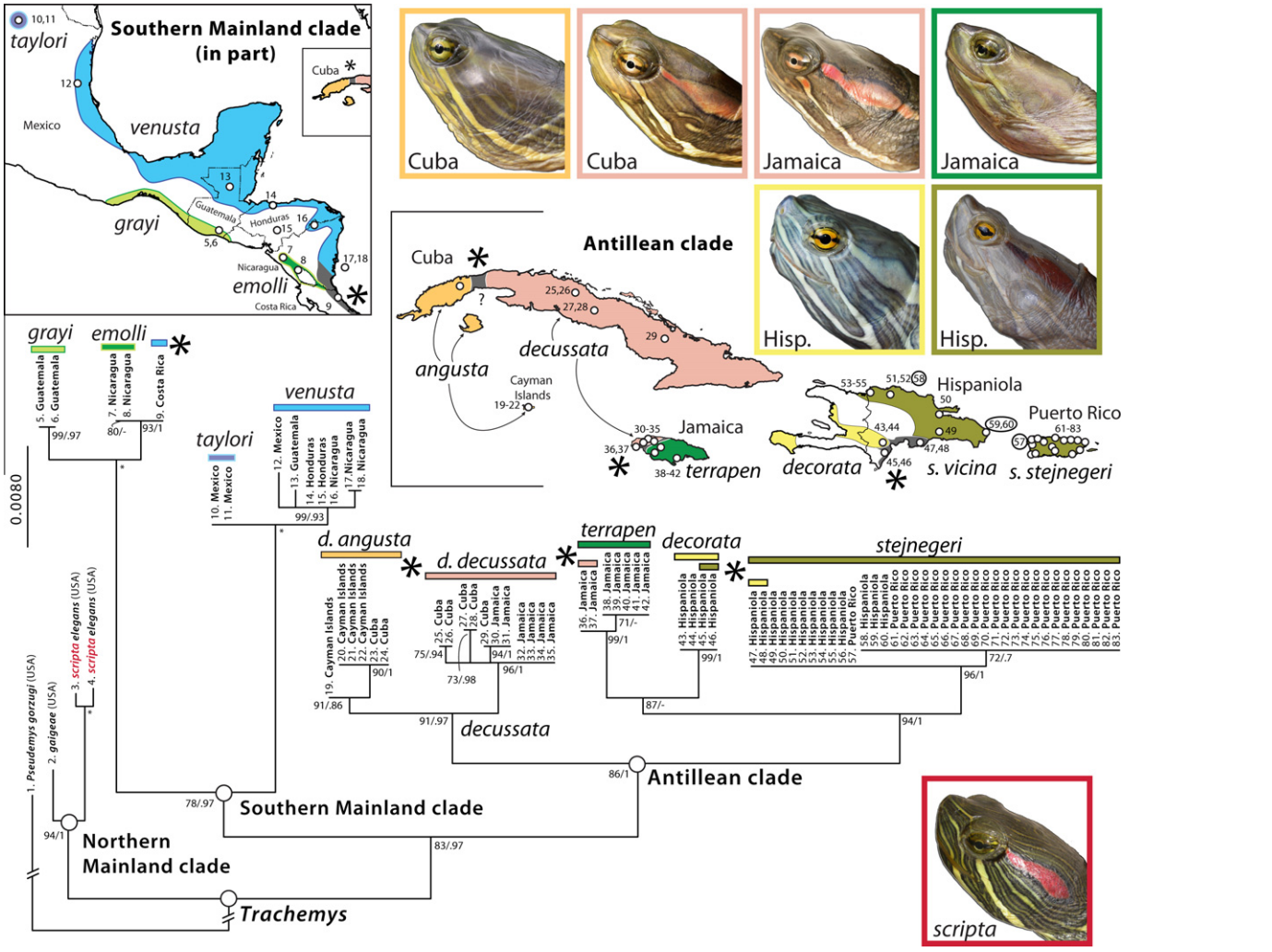

Fig. 1. Inset maps show the geographic distribution of relevant taxa and origin of Caribbean *Trachemys* samples used in this study (museum vouchers given in Appendix). Colors on the map, head images, and phylogenetic tree correspond. Asterisks (*) indicate areas of hybridization on the map and on the tree whereas circled samples 57–60 of *T. stejnegeri* may indicate translocations. Head images show morphological variation of Antillean *Trachemys* (upper right) and the invasive *Trachemys scripta elegans* (TSE, lower right). Phylogenetic tree is the analysis of the 83-sample mtDNA data set (908 bp, ND4). The tree is based on a RAxML likelihood analysis with likelihood bootstrap (left) and Bayesian posterior probabilities as support (right).

R35Ex2 (Fujita et al., 2004). For mitochondrial and nuclear markers, amplification reactions (25 μl) contained ~50 ng DNA, 1 X PCR buffer (20 mM Tris–HCl, 50 mM KCl), 1.5 mM $MgCl_2$, 0.4 μM each primer, 200 μM each dNTP, and 1.25 μ recombinant Taq DNA polymerase (Invitrogen). Thermal cycler profiles consisted of initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 54–63 °C for 30 s, and 72 °C for 1–2 min, with a final extension at 72 °C for 10 min. PCR products were then visualized on a 1% agarose gel and purified using ExoSAP-IT (USB, Cleveland Ohio). All samples were sequenced using the original PCR primers. Internal ND4 primers (L-ND4intEmys [TAGGCCTATGATGACTACTCG] and H-ND4intEmys [GAATGGCTATGTTGGCTAAGC]; new primers designed by B. Stuart) and internal R35 primers (L-R35int and H-R35int; Stuart and Parham, 2007) were also used for sequencing. Sequences were assembled and edited using Sequencher 4.7 (Gene Codes Corp.), BioEdit 7.0.9 (Hall, 1999) and Geneious 5.4 (Drummond et al., 2009). GenBank accession numbers for our new sequences and for sequences obtained from previous studies are provided (Appendix). All alignments were performed with MAFFT v6.814b (Katoh et al., 2002) followed by manual adjustments.

Each of the four ingroup alignments were tested for recombination using three tests implemented in Piganeau et al. (2004) and Piganeau and Eyre-Walker (2004): (1) Maximum Chisquare test of Maynard Smith (1992); (2) LDr2, the correlation between the measure of linkage disequilibrium, r2 (Hill and Robertson, 1968), and the distance between sites; (3) LDD', the correlation between the measure of linkage disequilibrium, D' (Lewontin, 1964), and the distance between sites. None of these tests detected recombination.

2.3. Mitochondrial analysis

We performed phylogenetic analyses of mitochondrial DNA that included all 83 samples examined in this study using Bayesian and Maximum Likelihood (ML) methods as implemented in MrBayes MPI version 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003; Altekar et al., 2004) and RAxML (Stamatakis, 2006) Pthreads version 7.2.6 (Ott et al., 2007), respectively. MrModeltest2 version 2.3 (Nylander, 2004) was used to estimate substitution models for the MrBayes analysis. The mitochondrial fragment was partitioned into the three coding positions for ND4 and one for the tRNAs. Under the Akaike information criteria (Akaike, 1973; Posada and Buckley, 2004) the HKY with equal rates model was selected for all partitions except the second codon position, which had a GTR with equal rates model applied. For the

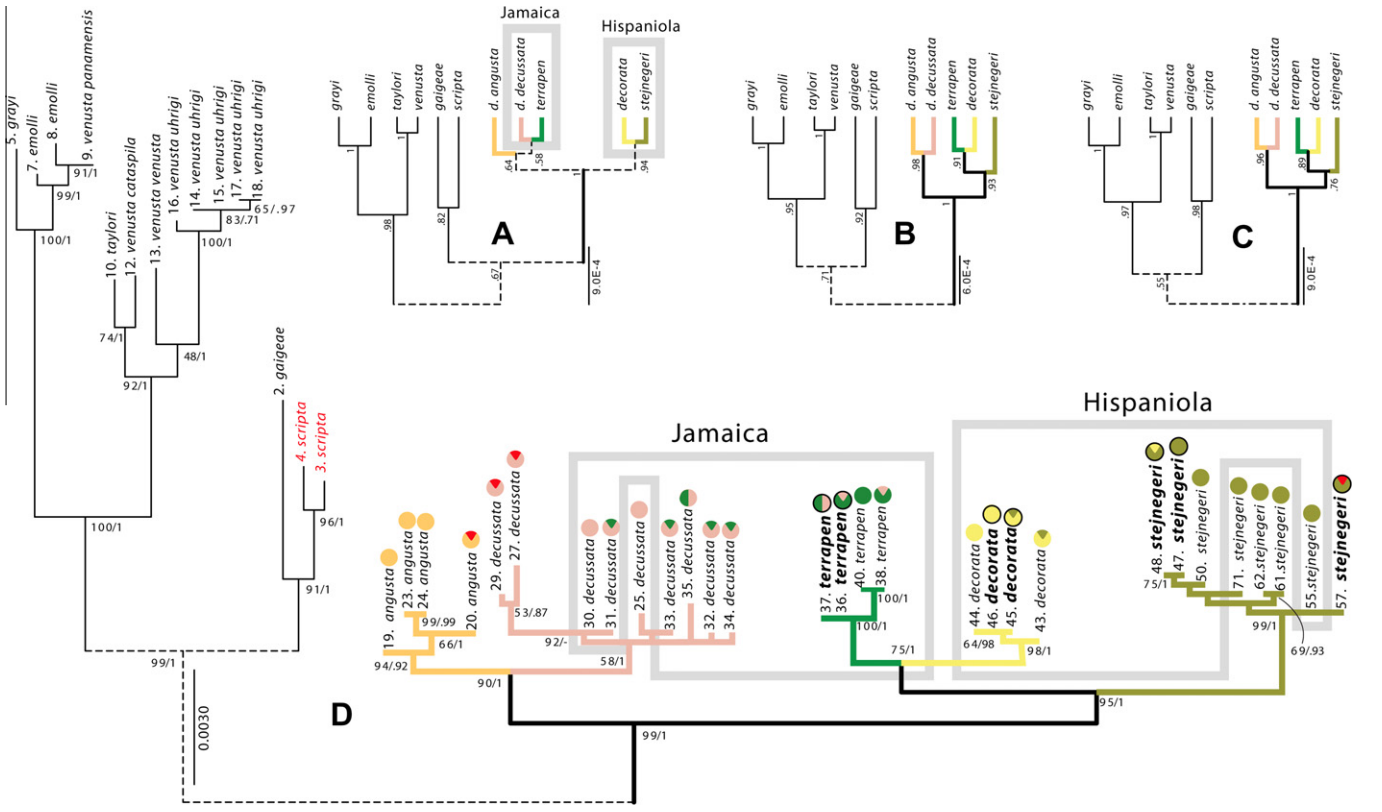

Fig. 2. Phylogenetic analyses of nuDNA and mtDNA. Taxa colors correspond to Fig. 1. Gray outlines indicate samples from Jamaica and Hispaniola where intergradation between parapatric species is observed. The dashed line leading to the outgroups indicates an uncertain topology (the furthest outgroup, *Pseudemys gorzugi*, removed for clarity of presentation). A–C: *BEAST Bayesian analyses of 12 "species" groups, including the five ingroup taxa from the Antilles and six *Trachemys* from the mainland. Node support values are posterior probabilities. A: *BEAST analysis including all phased alleles. Introgressed alleles shared between taxa on the same island yield an incorrect species tree (uncertainty of ingroup branches indicated by dashed lines). B: *BEAST analysis with all introgressed alleles removed. C: *BEAST analysis that does not include any data from phased alleles, i.e., all polymorphic sites are coded with International Union of Pure and Applied Chemistry (IUPAC) ambiguity codes. D: RAxML likelihood analysis of concatenated mtDNA and nuDNA data set with likelihood bootstrap (left) and Bayesian posterior probabilities as support (right). Pie diagrams for Antillean taxa show the presence of one or two introgressed alleles for the four sequenced markers. Morphological intergrades are shown with bold text and black outlines on the pie diagram.

RAxML analysis, the same 4 partitions were provided to RAxML for bootstrap and ML analyses under the GTRGAMMA option. This option independently estimates the alpha shape parameter of the Gamma model of rate heterogeneity, the empirical base frequencies, and the evolutionary rates in the GTR matrix for each partition.

2.4. Cloning and initial coalescent analysis

We also performed analyses on a subset of the 83 samples, which included 45 samples that were sequenced for three nuclear DNA markers. The chromatograms for these nuclear markers revealed multiple SNPs and length polymorphism for two R35, fifteen HNF-1α, and twelve RELN individuals. For the Caribbean sequences with two or more SNPs or length polymorphism we isolated individual alleles using the following cloning techniques. PCR products were directly ligated into the cloning vector pCR2.1-TOPO (Invitrogen) using the TOPO TA cloning kit and then transformed into Mach1-T1 chemically competent *E. coli* cells. Eight to 12 clones per sample were then picked, amplified using M13F and M13R primers, and then sequenced bidirectionally using the original marker PCR primers.

After cloning and assembling contigs, we noted the presence of shared alleles among *Trachemys* samples that belong to different species. This pattern suggests that ILS and/or hybridization may contribute to discordance between gene trees and species trees. To assess this issue we used *BEAST (v1.6.1) (Drummond and Rambaut, 2007) for a Bayesian inference of species trees from gene trees under a multispecies coalescent model (Heled and Drummond, 2010). This method incorporates multiple genes from multiple individuals per species to coestimate the gene trees embedded in a species tree. Our phased data allow us to treat each allele as an independent member of its designated "species." As a result, unique alleles from either incomplete lineage sorting or introgression are free to contribute to the calculation of the coalescent for that locus and potentially reveal their origins.

The *BEAST analysis included data from all four markers (three nuclear and one mitochondrial) assigned to 12 "species" groups with 113 alleles (61 for RELN, 47 for R35, 61 for HNF-1α, 45 for ND4). The "species" groups were based on mtDNA clades that are readily diagnosable based on morphological characters (Figs. 1 and 2). We used a relaxed uncorrelated lognormal clock model. For the site models, we used HKY with empirical base frequencies for both nuclear and mitochondrial partitions and assigned the site heterogeneity model to the proportion of invariable sites (propinv) to the nuclear partitions and invariable gamma (invgamma) to the mitochondrial partitions.

We ran eight independent analyses of 50 million generations with unique starting seeds sampling every 10,000 states for a total of 400 million generations and 40,000 trees. The log files from each analysis were combined using LogCombiner v1.6.1. Using Tracer 1.5 we examined the effective sample size for the combined analyses, determined an appropriate burnin for the sampled states, and confirmed stationarity around the likelihood mean. Each analysis produced a gene tree for each locus and a single species tree.

The trees for each locus were then combined with LogCombiner followed by annotation in TreeAnnotator 1.6.1. In LogCombiner, we removed (burnin) the first 500 trees of the 5000 trees (50 M/10,000 sampled states) generated for each of the eight analyses leaving a total of 36,000 trees for each gene and species tree file. This process produced a single log file, a single tree for each gene, and one species tree. Prior to each analysis we used MrModeltest2 (Nylander, 2004) to estimate the substitution model for each of the four markers and applied these models to the "Site Models" settings in BEAUTi v1.6.1.

2.5. Identification of potential introgressed alleles

We did not attempt to distinguish migration from isolation using applications that apply the Isolation with Migration model to molecular data, such as IM (Hey and Nielsen, 2004) and IMa2 (Hey, 2010). These programs require a priori estimates of divergence, assume that the populations being evaluated are the most recently diverged, and assume that no other un-sampled populations are exchanging genes with the sampled populations. An attempt at divergence dating *Trachemys* lineages (Fritz et al., 2011) relied on fossils that fall well short of best practices for justifying fossil calibrations (Parham et al., 2012) in terms of establishing their age or phylogenetic position. Additional study of *Trachemys* fossils may reveal justifiable calibrations but is outside the scope of this study. In addition to the absence of reliable divergence dates, there are many unknown and complex interactions among these populations that certainly violate the assumptions of IM and IMa2 analyses.

Instead, we performed exploratory phylogenetic analyses of individual alleles, a method that has proven successful in identifying hybrids (Stuart and Parham, 2007). First, we examined the coestimated 'gene' trees for each locus from the *BEAST analysis to look for obvious mismatches, i.e., alleles nested within clades composed primarily of alleles assigned to other 'species.' We found 28 potential mismatches in 16 samples (20 unique alleles; 13 HNF-1α, six RELN, one R35) that clustered with species that were different from their voucher's identification based on morphology and geography. To test for the potential hybrid origin of these 20 alleles, we performed a series of phylogenetic analyses based on the 45-sample data set that included the mitochondrial and three nuclear markers. First, we constructed an alignment for each mismatched allele and treated it as an independent OTU and applied ambiguity codes to SNPs of the remaining sequences. We then ran concatenated RAxML analyses that included the designation of partitions for each nuclear marker and the four mitochondrial partitions for a 50,000-replicate ML bootstrap and ML analyses under the GTR plus gamma option (GTRGAMMA). If the mismatched alleles were placed with samples of a different species, then we considered this further evidence of a hybrid origin, and refer to them as 'potential introgressed alleles' hereafter.

Once we identified potential introgressed alleles, they were excluded from a concatenated alignment that included all four markers. We ran likelihood bootstrap and likelihood analyses in RAxML and Bayesian phylogenetic analyses in MrBayes on this alignment using the same partitions and parameters as the previous analyses. The excluded introgressed alleles were mapped onto the resulting tree, indicating in which individuals they occurred (Fig. 2D).

2.6. Species tree comparisons

In the first *BEAST analysis (described above) we included all alleles, including those that were later identified as potential introgressed alleles. In order to assess the impact of introgressed alleles on the *BEAST estimation of species trees, we performed two additional *BEAST analyses. First, all introgressed alleles were removed (Fig. 2A). Using BEAUTi, we imported individual alignments for each 'gene' with introgressed alleles removed. This analysis did not include ambiguous characters for phased markers. We used the same *BEAST parameters as the previous analysis.

Most phylogenetic studies do not clone polymorphic markers; typically, ambiguous positions are converted to Ns or to the appropriate IUPAC ambiguity codes. To evaluate the influence of using ambiguous nucleotide coding versus actual nucleotides at SNP positions, we converted all SNPs to IUPAC ambiguity codes and imported a concatenated alignment of 45 OTUs with each gene identified as an independent gene. While we imported a concatenated alignment, *BEAST treats each partition as a unique gene and independently estimates each gene's coalescent and thus should not be considered a traditional "concatenated" phylogenetic analysis. This departs from our other *BEAST analyses, where we imported individual 'gene' alignments that included all phased alleles so that each alignment had unique numbers of OTUs and did not include ambiguous characters for phased markers. This final *BEAST analysis of ambiguous characters conceals any signal that might be generated by hybridization. The parameters for this *BEAST analysis were the same as above.

3. Results

3.1. Mitochondrial analysis

The RAxML and Bayesian analyses of mtDNA yielded similar trees, with just small differences among populations of a few subspecies (Fig. 1). Antillean samples form a clade, as has been suggested by other studies that were focused on other clades of *Trachemys* (Jackson et al., 2008; Fritz et al., 2011). The maximum uncorrected sequence divergence (usd) among Antillean samples is less than 1.9% (Supplement 1). The recovery of two major clades in the outgroup samples, the 'Northern and Southern Mainland Clades', also matches the topology of previous studies. The relationship of these two clades to the Antillean clade, however, varies among our analyses and is not well supported by any study. Within the Southern Mainland Clade there is a relatively deep division (>3.7% usd) between a clade that includes *T. venusta* and *T. taylori* (which are just 1 bp different from one another) and a clade that includes *T. grayi* and *T. emolli* (<0.5% usd). This division largely corresponds to geography with one exception (Fig. 1). Our sample from Costa Rica, assigned to *T. venusta* based on geography, was found to be identical to *T. emolli*. The significance of this result is treated in the discussion.

Within the Antillean clade, *T. decussata* is sister taxon to a clade comprising the remaining taxa. A monophyletic *T. d. angusta* is characterized by overall low mtDNA diversity, with the exception of one anomalous sequence from the Cayman Islands (sample 19). The mtDNA confirms that samples identified from Jamaica are *T. d. decussata.* The identity of the northern Jamaican populations has been in question since Tuberville et al. (2005) noted their morphological distinctiveness and speculated that they may be hybrids with *T. decussata*, *T. stejnegeri*, or *T. scripta*. The Jamaican *T. decussata* samples are clearly related to their Cuban conspecifics, as indicated by the morphology, mtDNA, and nuDNA of the samples. The Jamaican samples include two mtDNA haplotypes, one of which matches that from a sample on Cuba. Cuban *T. d. decussata* show different haplotypes for each sampled locality, and this may indicate geographic structure to the genetic variation among populations.

Our *T. terrapen* samples are represented by two mtDNA haplotypes (1 bp different) from four localities that span about half of the island of Jamaica. This pattern indicates a low overall mitochondrial diversity for this species. *Trachemys terrapen* is the sister

taxon to *T. decorata* (<0.8% usd) from Hispaniola, which is represented by a single haplotype in our samples. Both *T. terrapen* and *T. decorata* include morphological intergrades with other species. In the case of *T. decorata*, there are four intergrades. The two intergrades closest to the range of pure *T. decorata* have *T. decorata* mtDNA, whereas the two intergrades that are closest to the range of pure *T. stejnegeri* have *T. stejnegeri* mtDNA. *Trachemys stejnegeri* has two haplotypes (1 bp different) that largely correspond to the islands of Hispaniola and Puerto Rico, and hence the named subspecies *T. s. vicina* and *T. s. stejnegeri*, respectively. Three of the four cases of non-correspondence occur where the islands are closest to one another.

3.2. Initial coalescent analysis and identification of potential introgressed alleles

The *BEAST analysis including the 29 cloned alleles recovered a topology (Fig. 2) that differs from the mtDNA tree in the following ways: For one, the relationship of the *Trachemys* outgroups is different; in the *BEAST species tree the Northern Mainland Clade is found to be the sister taxon to the Antillean clade, whereas in the mtDNA haplotype tree the Southern Mainland clade is the nearest outgroup. We do not consider this result significant given that the support value is low and that confidently rooting these outgroups requires much additional study of the genus as a whole. More importantly, the *BEAST analysis recovers a novel relationship of ingroup taxa. *Trachemys decussata* is found to be paraphyletic to *T. terrapen,* and *T. stejnegeri* is found to be the sister taxon to *T. decorata*. For these species, the *BEAST analysis more closely matches geography because taxa that inhabit the same island are found to be each other's closest relative. The combined presence of intermediate individuals and a problematic species tree suggest hybridization among the sampled lineages.

Coestimated gene trees from this coalescent analysis were examined to identify 28 potential mismatches in 16 samples (20 unique alleles; 13 HNF-1α, six RELN, one R35). The phylogenetic analysis of these alleles showed that 17 mismatches in 15 samples are plausibly the result of hybridization (12 unique alleles; seven HNF-1α, 4 RELN, one R35). Two of these 17 potential introgressed alleles were found in Cuban samples but show an affinity to TSE alleles, a result with no simple explanation. However, most of these potential introgressed alleles (15 of 17) occur near hybrid zones evinced by intergrade samples or likely TSE invasion sites. We consider this pattern further evidence of their hybrid origin and the moderate success of our heuristic method to identify allelic evidence of hybridization. All 17 of the potential introgressed alleles are mapped onto a concatenated analysis of mtDNA and nuDNA, which has a topology that largely matches the mtDNA analysis (Fig. 2D).

3.3. Species tree comparisons

The *BEAST Bayesian analysis with all alleles recovered a species-tree topology that conflicted with the mtDNA tree and traditional taxonomy (Fig. 1) (see 3.2). To test whether the potential introgressed alleles were confounding this analysis we performed additional *BEAST analyses that excluded potential introgressed alleles or else masked the signal of all polymorphisms with IUPAC ambiguity codes. These analyses recovered species tree topologies that were more similar to those found in the mtDNA and concatenated analyses (Figs. 2B and C). Specifically, *T. decussata* is recovered as monophyletic and *T. terrapen* and *T. decorata* are resolved as sister taxa.

The main differences between the analysis with all potential introgressed alleles removed (Fig. 2B) and the analysis with polymorphisms scored with IUPAC ambiguity codes (Fig. 2C) is a reduction in support values for the latter. This is logical since the variation at all sites with potential introgressed alleles would be masked, including any valid phylogenetic signal. The confounding impact of the potential introgressed alleles on the initial coalescent analysis, combined with the comparison of support values between the other two analyses, suggests that the potential introgressed alleles confound the *BEAST Bayesian inference of a species tree from multiple markers, which we interpret as further support for their hybrid origin.

4. Discussion

4.1. Evidence for hybridization and translocation

Antillean *Trachemys* are a recent radiation with relatively low genetic variability among populations (maximum raw mt sequence divergence of <1.9%). Despite widespread ILS among our samples, we observe patterns of shared alleles that are consistent with hybridization by analyzing the phylogenetic position of taxonomically inconsistent alleles. Our data, combined with data from the literature (see Section 4.1.4) identify four contact zones in the Caribbean, three in the Antilles (Jamaica, Hispaniola, Cuba) and one on the mainland (Central American Isthmus). The Jamaican and Central American contact zones are recognized here for the first time. Previous studies have shown two other instances of hybridization or intergradation within the Northern Mainland clade in the USA (Seidel et al., 1999). We hypothesize that species of *Trachemys* are likely to interbreed whenever they come into contact.

The patterns and degrees of hybridization likely vary among *Trachemys* contact zones. Our data are too preliminary to compare these differences in detail, but we can show repeated discordance between obvious morphological characters and allelic introgression. Because of backcrossing, the genetically identified descendants of hybrids may not show characters of both the parents. None of our samples are F1 hybrids, meaning that they are backcrossed and do not represent isolated hybridization events. More detailed studies of how alleles and morphological characters migrate across these contact zones would help to explain natural patterns and processes of turtle evolution, a group that is known for lineage reticulations (Spinks et al., 2004; Buskirk et al., 2005).

We outline the evidence for hybridization for each region within the study area below. We also provide evidence and hypotheses relating to the human translocation of *Trachemys* species that may be the source of the contact.

4.1.1. Jamaica

Our data show that *Trachemys* populations in northwest Jamaica are *T. decussata*, a taxon that was previously known to occur only on Cuba and associated islands (Fig. 1). Jamaica was thought to have a single species, *T. terrapen*, but a recent field survey showed the presence of different-looking turtles in the northwest that were speculated to be hybrids between *T. terrapen* and *T. decussata*, *T. stejnegeri*, or *T. scripta* (Tuberville et al., 2005). Our samples from this area suggest that *T. decussata* genetics and morphotypes dominate, but also show evidence for hybridization, including the transfer of alleles and the presence morphological intergrades (Fig. 2D). Samples 30–34 were not considered morphological intergrades, but that may be because variation within populations of either species is not well understood, and so our criteria are necessarily coarse grained. Further genetic sampling accompanied by quantifiable morphological analyses of the new samples and museum specimens can elucidate this pattern.

The occurrence of *T. decussata* on Jamaica is not easily explained because we cannot distinguish a natural dispersal event from prehistoric human-mediated dispersal. Our data show that Jamaica

has more than one mtDNA haplotype, including some (samples 30, 31) that match a haplotype (sample 29) from Cuba. Therefore, dispersal could be very recent and within the range of human history in the region. The Taíno people that inhabited this region prior to European contact were known to use turtles as a resource (Alvarez, 1994; deFrance and Newsom, 2005; Carlson and Steadman, 2009) and to introduce food species to islands (deFrance and Newsom, 2005; Giovas et al., 2011). Turtles are a readily transportable source of protein and have been aptly described as 'living cans' (Vamberger et al., 2011). The transport and origin of turtle populations have been attributed to prehistoric human activity in other parts of the world (Vamberger et al., 2011) and undoubtedly occurred in the Caribbean. Unraveling whether Taínos or ocean currents brought *T. decussata* to Jaimaica has important conservation implications, especially as it pertains to the contact zone between *T. decussata* and *T. terrapen*. The different possible origins of *T. decussata* will dictate whether this contact zone is a natural phenomenon or the introgression of alleles into an endangered endemic from a human-introduced invasive species within the past 1400 years (the age of the earliest human occupants of Jamaica [Callaghan, 2008; Rampersad, 2009]). Additional data from Cuba and Jamaica are needed to shed light on the origins of Jamaican *T. decussata*. It is important to clarify the status of this species and its contact zone with *T. terrapen* because of the serious conservation situation of turtle populations on Jamaica (Wilson, 2011).

4.1.2. Hispaniola

Our data confirm a contact zone between *T. decorata* and *T. stejnegeri* on Hispaniola that was suggested by previous authors (Seidel and Inchaustegui Miranda, 1984) based on the presence of morphological intergrades. In this case, the contact between these two species appears to result from a natural overlap of the species' geographic distributions. We sampled *T. decorata* from two localities; both localities showed some genetic influence from *T. stejnegeri*, and one showed morphological evidence of intergradation onto the Pedernales Peninsula. It is possible that much of the range of *T. decorata* in the Dominican Republic contains genes from *T. stejnegeri*, and that genetically pure populations are restricted to Haiti. Unfortunately, the conservation status of Haitian *T. decorata* is likely to be very poor given the nearly complete destruction of the native herpetofauna there (Wilson et al., 2006; Hedges and Díaz, 2011).

In addition to the contact zone between *T. decorata* and *T. stejnegeri*, we note a possible secondary contact between *T. stejnegeri* from Hispaniola and Puerto Rico. Our mitochondrial analysis identifies two mt haploclades that would seem to support the recognition of the traditionally recognized subspecies (*T. s. stejnegeri* on Puerto Rico and *T. s. vicina* on Hispaniola). Just four of our 36 samples of *T. stejnegeri* conflict with this pattern, but three of those are from either the easternmost sampled locality of Hispaniola or the westernmost locality of Puerto Rico. This distribution of mt haplotypes suggests occasional two-way dispersal of *T. stejnegeri*, but as with the situation described for Jamaica above, it is unclear whether this dispersal was by natural means or human activity. However, the hypothesis that this genetic pattern is caused by human translocation of turtles is supported by the fact that the Taínos on the eastern tip of Hispaniola and western Puerto Rico had daily contact and engaged in extensive trading (de las Casas, 1951; Rouse 1948, 1986).

4.1.3. Puerto Rico

In addition to the possible connection with Hispaniola described above, our data suggest that feral TSE may be hybridizing with native freshwater turtles on Puerto Rico. Of the four Puerto Rican samples for which we sequenced nuDNA, one (57) showed an allele that is unlike those in any other Antillean samples. A phylogenetic analysis placed this allele among mainland samples, although the exact placement is unclear. This sample came from a pond that included invasive TSE and also exhibited some morphological characters of TSE. We suggest that this specimen is a back-crossed hybrid.

Of the four Greater Antilles, Puerto Rico is the most susceptible to a TSE invasion and subsequent genetic pollution of native populations because of a combination of factors. For one, unlike the other three islands, turtles are still common on Puerto Rico because they are not routinely harvested for food. On Cuba, Hispaniola, and Jamaica, turtles are heavily utilized as a food resource and so populations are much reduced. Presumably any feral TSE would be eliminated by the same unsustainable harvest that impacts the native species. Second, there is a sufficient pet trade on Puerto Rico to provide a source for feral TSE populations. On Cuba, Hispaniola, and Jamaica the pet trade is not as well developed and restricted to urban areas. Combined with the first factor, it is therefore highly unlikely that TSE and native species would ever meet. Whatever pet trade does exist is centered on major cities whereas the surviving native populations are restricted to relatively remote rural regions. Finally, Puerto Rico is small, meaning that any feral TSE population can easily spread to all parts of the island. The observations of TSE from different parts of Puerto Rico suggest that this invasion has already occurred (Joglar et al., 2007).

4.1.4. Cuba and the Cayman Islands

Cuba is the largest of the four Greater Antilles, and the place where we have the fewest sampled localities (Fig. 1). Therefore, our ability to describe genetic diversity of Cuban *Trachemys* is limited. A presumed intergrade zone exists between *T. d. angusta* in the west and *T. d. decussata* in the east (Fig. 1), but it is virtually unstudied. The only record of this intergradation is from a 70-year-old mention of intermediate individuals (Barbour and Carr, 1940). We have no reason to doubt that these two taxa contact and interbreed, but the extent and distribution of this intergrade zone are completely unknown. This intergradation was used to place *T. d. angusta* as a subspecies of *T. decussata* (Barbour and Carr, 1940) where it has remained ever since (Seidel, 1988; Seidel, 2002; Turtle Taxonomy Working Group, 2007b, 2011). However, we argue that the presence of hybrids, or even a small intergrade zone, is not a sufficient argument for assigning conspecific status. Our caveat is especially relevant given the occurrence of such contact zones on Jamaica and Hispaniola. Moreover, the data from our samples show that, in addition to being morphologically distinct, *T. d. decussata* and *T. d. angusta* are separate genetic lineages. These data suggest that *T. d. angusta* could be elevated to full species status, but we refrain from making this recommendation following our philosophy of taxonomic conservatism (see 4.3.) and pending studies with more intensive sampling.

One sample of *T. d. angusta* from the Cayman Islands shows evidence of introgression from *T. scripta*. The situation for the Cayman Islands is very similar to that of Puerto Rico in that there is an active pet trade, wild turtles are not intensely harvested, and the overall area is small. As with Puerto Rico, TSE is common on the Cayman Islands and so it is plausible that there is genetic influence from this invasive species. In fact, hybrids of TSE and *T. d. angusta* have been reported from the Cayman Islands already (Lever, 2003). The conservation implications of this finding are complicated by the plausible hypothesis that *T. d. angusta* is not native to the Cayman Islands (Seidel and Franz, 1994).

In contrast to the Cayman Islands, the presence of putative *T. scripta* alleles on Cuba (Fig. 2D) is more difficult to explain because Cuba lacks all of the characteristics that would lend itself to a TSE invasion. There is no active or historically documented pet trade, most wild turtles are actively harvested, and the island is relatively large. There are no documented occurrences of feral TSE on Cuba,

even near the cities, and all of our samples are from relatively rural areas (Fig. 1). For all of these reasons we are reluctant to accept feral TSE as the origin of *T. scripta* alleles in Cuba. We are also skeptical of natural dispersal or prehistoric translocation of *T. scripta* to Cuba. The Florida Current creates one of the strongest biogeographic and cultural barriers in the New World. High levels of endemism characterize the natural fauna of the West Indies, and none of its nonvolant terrestrial vertebrate fauna are thought to have crossed the Straits of Florida from the North (Hedges, 2001, 2006a). Secondly, pre-Columbian trade between Cuba and the mainland was sporadic at best (Callaghan, 2010) and aside from very recently introduced populations there are no records of *T. scripta* from southern Florida, including the archaeological record.

A final possibility is that the putative introgressed alleles of Cuban *Trachemys* are not from *T. scripta* at all, but are an artifact of ILS. We are similarly reluctant to favor this hypothesis because our method of identifying hybrids seems to be effective, showing a signal consistent with contact zones. Finally, in the case of the *T. decussata* samples, the introgressed alleles show a strong similarity to those in *T. scripta* (e.g., the HNF allele of TSE sample 3 is identical to that of *T. decussata* sample 20 and one bp different from sample 29). As genetic data sets for *Trachemys* become more comprehensive in their sampling of populations and markers, we should be able to explain this unusual finding.

4.1.5. Central America

At the southern end of our sampling of *T. venusta* we include one sample from Costa Rica. The DNA from this sample is very similar to our *T. emolli* samples. A recent study also found a sample of *T. venusta* from "Panama" that shows a strong genetic affinity with *T. emolli* (Fritz et al., 2011). The Atlantic lowlands of southern Nicaragua and Costa Rica are where the Río San Juan connects *T. emolli* populations around Lago Nicaragua to populations of typical *T. venusta* (Legler, 1990; Fig. 1). Therefore we suggest that this area represents a zone of secondary contact and ongoing intergradation, possibly stretching south to Panama.

4.2. Impact of introgressed alleles on species tree coalescent analyses

The *BEAST multispecies coalescent analysis that included all phased alleles recovers a species tree that conflicts strongly with the mitochondrial DNA tree (Figs. 1 and 2A). The main source of conflict relates to the phylogenetic position of hybridizing taxa. In the "all alleles" analysis, hybridizing taxa are found to be sister taxa, such that clades more closely approximate geography. In other words, the two species on Jamaica are sister taxa and the two taxa on Hispaniola are sister taxa. After removing all of the 17 potential introgressed alleles, the topology of the *BEAST species tree (Fig. 2B) resembles that of the mtDNA tree where species on the same island are not necessarily sister taxa. We hypothesize that alleles shared by hybrids yield an incorrect species tree, uniting taxa that are not actually sister taxa.

To test this hypothesis we ran a third coalescent analysis where each polymorphic site was coded as ambiguous. In this analysis the effect of hybridization is largely masked from the alignment, mimicking an alignment made from non-phased sequences. The resultant topology (Fig. 2C) matches the mitochondrial tree and coalescent analysis with potential introgressed alleles removed, and we take this as further support for our hypothesis that hybridization confounds species-tree estimation.

The identification of introgressed alleles, while seemingly effective in this case, is highly dependent on sampling, assumptions, and is very time consuming (non-automated). The heuristic analysis of the phylogenetic positions of alleles gave plausible results here, but we recognize that there are factors that could render this approach less useful in other cases. For example, under-sampling allelic variation or the sampling/presence of many highly introgressed individuals relative to 'pure' stock would make the identification of introgressed alleles problematic. Importantly, we were able to compare our results to the geographic distribution and morphology of our samples, but this may not be possible for other studies that rely on samples of uncertain provenance or that lack vouchers.

Cloning polymorphic samples helped to illuminate patterns of introgression among Antillean *Trachemys*. However, the fact that the analysis without phased alleles recovers the preferred tree shows that it may not be helpful to phase sequences if the goal is to estimate the species tree and not to use coestimated gene trees to identify hybridization. The topology of the ambiguous sequence analysis (Fig. 2C) completely matches the results from the analysis with the introgressed alleles removed (Fig. 2B). The only difference is a decrease in support values in the ambiguous sequence analysis resulting from synapomorphic nucleotide positions being obscured by ambiguity codes.

4.3. Taxonomy of Trachemys

Although the focus of our study is on Antillean *Trachemys*, we also sequenced samples of adjacent mainland populations in order to test for the possibility that they contributed to the genetic composition of Antillean populations. As a side product, our sampling was dense enough to give some insights into the genus beyond the Greater Antilles.

Our analyses recover the three main lineages of *Trachemys* found by previous authors (Jackson et al., 2008; Fritz et al., 2011): (1) Antillean *Trachemys* (the ingroup, six taxa from the Greater Antilles); (2) The Northern Mainland clade (five taxa from USA and northernmost Mexico); and (3) The diverse Southern Mainland clade (15 taxa ranging from Mexico through South America). The relationship among these three major clades of *Trachemys* (Antillean *Trachemys*, Southern Mainland clade, Northern Mainland clade) is not well established by any study, including ours (Jackson et al., 2008; Fritz et al., 2011; Figs. 1 and 2). We consider the resolution of these three clades to be an open question.

The Southern Mainland clade of *Trachemys* includes deeper, older lineages than do either the Northern Mainland clade or West Indian *Trachemys*, but the taxonomy of these lineages is in flux. A relatively stable nomenclature based on a phylogenetic analysis of morphological characters (Seidel, 2002; Turtle Taxonomy Working Group, 2011) has recently been modified by the description of new subspecies (McCord et al., 2010), as well as by a genetic analysis that made sweeping nomenclatural recommendations (Fritz et al., 2011). The most significant genetic finding is that a taxon from the Pacific coast of Mexico (*T. ornata*) is closely related to and likely conspecific with taxa (*T. venusta* and subspecies) from the Atlantic coast of Mexico and Central America (Fritz et al., 2011). In addition to the surprising biogeographic pattern, this result would necessitate that the six eastern subspecies traditionally associated with the epithet *Trachemys venusta* (Gray, 1856) should now be transferred to the older name *Trachemys ornata* (Gray, 1830). We do not have any samples of *T. ornata* in our study, and the samples of the other study are derived from an introduced population near the resort town of Acapulco (Legler, 1990; Seidel, 2002; Fritz et al., 2011). Such feral populations may include more than one taxon of interbreeding turtles, thereby confounding genetic results. Even though we feel that the results of the previous study are likely correct, given the significance of this taxonomic change and the extensive name changes it would generate, the unusual biogeographic scenarios it would necessitate, and the suboptimal samples at hand, we tentatively retain the traditional taxonomy pending comparisons of *T. venusta* and Acapulco samples with a study of natural populations of *T. ornata*.

A previous genetic study (Fritz et al., 2011) showed that *T. venusta grayi* was not part of the *T. venusta* complex and so should be recognized as a distinct species, *T. grayi*. We accept this taxonomic change, but also retain *T. emolli* as a distinct species separate from *Trachemys grayi* (Bocourt, 1868). *Trachemys emolli* (Legler, 1990) is allopatric and diagnosable with morphological and molecular characters as a separate lineage from *T. grayi*. *Trachemys taylori* was not included in that previous study but is clearly close to the *T. venusta* complex (Jackson et al., 2008; Figs. 1 and 2). *Trachemys taylori* is recovered as the sister taxon to *T. venusta* samples in mitochondrial trees, but they differ by only a single bp. When nuclear data are included (Fig. 2) our sample of *T. v. venusta* is weakly placed as the sister taxon to *T. taylori*. Acknowledging this weak genetic differentiation and the discrepancy between our results and current taxonomy, we maintain the morphologically and molecularly diagnosable *T. taylori* as separate from *T. venusta* pending additional study.

We support a conservative approach to taxonomic changes (Parham et al., 2006; Turtle Taxonomy Working Group, 2007a,b; Spinks et al., 2009b), and note that many of the suggested changes of Fritz et al. (2011) would necessitate adopting idiosyncratic criteria for recognizing species or subspecies based on percent sequence divergence. From our point of view, congruent patterns of morphological and genetic variation (multiple properties of species) should carry more weight than the arbitrary cutoff of a single criterion (de Queiroz, 2007), especially when detailed studies with dense geographic sampling are lacking in most areas. After all, speciation can occur rapidly (Seehausen, 2006) and without monophyletic gene trees (Knowles and Carstens, 2007). Combined with the fact that hybridization is common within turtles (Parham et al., 2001; Buskirk et al., 2005; Stuart and Parham, 2007), we urge caution against the over-interpretation of preliminary results.

5. Conclusions

The species tree comparisons combined with the morphology and geographic distribution of samples with mismatched alleles strongly suggest hybridization among lineages of Antillean freshwater turtles. Data from mainland *Trachemys* (Central America and the USA) presented here and elsewhere show that hybridization likely occurs whenever two *Trachemys* taxa contact. In Puerto Rico, where the invasive pet shop turtles, TSE, are common they are already interbreeding with the endemic species. Hybridization between TSE and an Antillean species is also occurring in the Cayman Islands, although the conservation implications are complicated by the likely non-native status of *T. d. angusta* there. The Cayman situation highlights the fact that some Antillean *Trachemys* populations result from human-mediated dispersal. In some cases, the impact is minor, such as the prehistoric translocation of genetically similar subspecies of *T. stejnegeri* between Puerto Rico and Hispaniola. In other instances, such as the possible translocation of *T. decussata* from Cuba to Jamaica, the implications are more serious. Whether the hybridization of these distinct species on Jamaica is the result of natural phenomena or human introduction will affect conservation decisions about Jamaican *Trachemys*. The sequencing of more markers from these and additional samples from the Greater Antilles will test the hypotheses presented here and shed new light on some of the lingering issues, especially explanations for the origin of Jamaican *T. decussata* and the presence of TSE-like alleles on Cuba.

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Appendix A

Vouchers for genetic sequences (GenBank Accesion numbers JN707346-JN707597) used in this study. Museum abbreviations: CAS, California Academy of Sciences Herpetology Collection, San Francisco, California, USA; CZACC, Colecciones Zoológicas del Instituto de Ecología y Sistemática, Havana, Cuba; IBH, Instituto de Biología Herpetology Collection, Universidad Nacional Autónoma de México, Mexico City, Mexico; IJZ R, Institute of Jamaica Reptile Collection, Kingston, Jamaica; MNHN, Museo Nacional de Historia Natural, Santo Domingo, Dominican Republic; MHUL, Museo Herpetológico de la Universidad Nacional Autonoma de Nicaragua, León, Nicaragua; MVZ, Museum of Vertebrate Zoology, University of California, Berkeley, USA; SMF, Naturmuseum Senckenberg, Frankfurt, Germany; TCWC, Texas Cooperative Wildlife Collection, Texas A&M University, College Station, Texas, USA; FLMNH, Florida Museum of Natural History, University of Florida, Gainesville, Florida, USA. Vouchers are whole-body preserved specimens except for three outgroup specimens, indicated below (9–11). Outgroups (1–18); 1. *Pseudemys gorzugi*. New Mexico, USA (MVZ 265706); 2. *Trachemys gaigeae*. Texas, USA (MVZ 265710); 3. *Trachemys scripta elegans*. Texas, USA (TCWC 68553); 4. *T. scripta elegans* Texas, USA (MVZ 265717); 5. *Trachemys grayi*. Departamento de Santa Rosa, Guatemala (MVZ 263975); 6. *T. grayi*. Departamento de Santa Rosa, Guatemala (MVZ 263976); 7. *Trachemys emolli*. Departamento de

Granada, Nicaragua (MVZ 263793); 8. *T. emolli.* Departamento de León, Nicaragua (MVZ 263795); 9. *Trachemys venusta*. Costa Rica (FLMNH 37160, skeletonized); 10. *Trachemys taylori*. Coahuila, Mexico (MVZ Obs Herp 5, photographic voucher); 11. *T. ta*ylori. Coahuila, Mexico (MVZ Obs Herp 4, photographic voucher); 12. T. venusta. Tamaulipas, Mexico (IBH 25690); 13. *T. venusta*. Departamento de Petén, Guatemala (MVZ 264176); 14. *T. venusta.* Departamento de Atlántida, Honduras (MVZ 263395); 15. *T. venusta*. Departamento de Francisco Morazán, Honduras (FLMNH 150886); 16. *T. venusta*. Departamento de Jinotega, Nicaragua (SMF 88199); 17. *T. venusta*. Región Autónoma del Atlántico Sur, Nicaragua (MHUL 009); 18. *T. venusta.* Región Autónoma del Atlántico Sur, Nicaragua (MHUL 010). Cuba and Cayman Islands (19–29); 19. *Trachemys d. angusta.* Cayman Islands (MVZ 265781); 20. *T. d. angusta.* Cayman Islands (MVZ 265779); 21. *T. d. angusta.* Cayman Islands (MVZ 265780); 22. *T. d. angusta.* Cayman Islands (MVZ 265782); 23. *T. d. angusta.* Provincia de Pinar Del Rio, Cuba (CAS 244120); 24. *T. d. angusta.* Provincia de Pinar Del Rio, Cuba (MVZ 265771); 25. *Trachemys decussata.* Provincia de Villa Clara, Cuba (CZACC 4.12777, MVZ 265760); 26. *T. decussata.* Provincia de Villa Clara, Cuba (CZACC 4.12778, MVZ 265761); 27. *T. decussata.* Provincia de Sancti Spíritus, Cuba (CZACC 4.12776, MVZ 265757); 28. *T. decussata.* Provincia de Sancti Spíritus, Cuba (CAS 244117); 29. *T. decussata.* Provincia de Camagüey Province, Cuba (CAS 244116). Jamaica (30–42); 30. *T. decussata.* Trelawney Parish (CAS 244108); 31. *T. decussata.* Trelawney Parish (CAS 244109); 32. *T. decussata.* Hanover Parish (CAS 244105); 33. *T. decussata.* Hanover Parish (CAS 244106); 34. *T. decussata.* St. James Parish (IJZ 260); 35. *T. decussata.* St. James Parish (CAS 244107); 36. *Trachemys decussata/terrapen.* Westmoreland Parish (CAS 244103); 37. *T. decussata/terrapen.* Westmoreland Parish (CAS 244104); 38. *Trachemys terrapen.* St. Elizabeth Parish (CAS 244101); 39. *T. terrapen.* St. Elizabeth Parish (CAS 244102); 40. *T. terrapen.* St. Catherine Parish (CAS 244098); 41. *T. terrapen.* St. Catherine Parish (CAS 244099); 42. *T. terrapen.* Manchester Parish (IJZ R259). Dominican Republic, Hispaniola (43–56, 58–60); 43. *Trachemys decorata.* Provincia de Barahona (CAS 238932); 44. *T. decorata*. Provincia de Barahona (MNHN 23.873-10); 45. *Trachemys decorata/stejnegeri.* Provincia de Pedernales (MNHN 23.828-10); 46. *T. decorata/stejnegeri.* Provincia de Pedernales (CAS 238931); 47. *T. decorata/stejnegeri.* Provincia de Peravia (CAS 238940); 48. *T. decorata/stejnegeri.* Provincia de Peravia (CAS 238941); 49. *Trachemys stejnegeri.* Distrito Nacional (CAS 238937); 50. *T. stejnegeri.* Borders of Provincias de Samana and Duarte (CAS 238933); 51. *T. stejnegeri.* Vuelta Larga, Provincia de Puerto Plata (CAS 238936); 52. *T. stejnegeri.* Maimon, Provincia de Puerto Plata (MNHN 23.876.10); 53. *T. stejnegeri.* Carbonera, Provincia de Monte Cristi (MNHN 23.874-10); 54. *T. stejnegeri.* Provincia de Monte Cristi (MNHN 23.875.10); 55. *T. stejnegeri.* (CAS 238934); Provincia de Monte Cristi; 56. *T. stejnegeri.* (CAS 238935); Provincia de Monte Cristi; 57. See Puerto Rico section after 60; 58. *T. stejnegeri.* El Cupey, Provincia de Puerto Plata (MNHN 23.877-10); 59. *T. stejnegeri.* Provincia de Altagracia (CAS 238938); 60. *T. stejnegeri.* Provincia de Altagracia (CAS 238939); Puerto Rico (57, 61–83); 57. *T. stejnegeri/scripta?* Municipio de Añasco (CAS 249622); 61. *T. stejnegeri.* Municipio de Añasco (CAS 249623); 62. *T. stejnegeri.* Municipio de Aguada (CAS 249625); 63. *T. stejnegeri.* Municipio de Camuy (CAS 249626); 64. *T. stejnegeri.* Municipio de Hatillo (CAS 249627); 65. *T. stejnegeri.* Municipio de Manatí (MVZ 265693); 66. *T. stejnegeri.* Municipio de Toa Baja (CAS 249618); 67. *T. stejnegeri.* Municipio de Toa Baja (CAS 249619); 68. *T. stejnegeri.* Municipio de San Juan (MVZ 265701); 69. *T. stejnegeri.* Municipio de San Juan (MVZ 265801); 70. *T. stejnegeri.* Municipio de Loiza (CAS 249628); 71. *T. stejnegeri.* Municipio de Fajardo (CAS 249614); 72. *T. stejnegeri.* Municipio de Fajardo (CAS 249615); 73. *T. stejnegeri.* Municipio de Humacao (CAS 249616); 74. *T. stejnegeri.* Municipio de Humacao (CAS 249617); 75. *T. stejnegeri.* Municipio de Patillas (MVZ 265699); 76. *T. stejnegeri.* Municipio de Patillas (MVZ 265700); 77. *T. stejnegeri.* Municipio de Juan Díaz (MVZ 265697); 78. *T. stejnegeri.* Municipio de Juan Díaz (MVZ 265698); 79. *T. stejnegeri.* Municipio de Jayuya (CAS 249620); 80. *T. stejnegeri.* Municipio de Jayuya (CAS 249621); 81. *T. stejnegeri.* Municipio de Lajas (MVZ 265694); 82. *T. stejnegeri.* Municipio de Cabo Rojo (MVZ 265695); 83. *T. stejnegeri.* Municipio de Cabo Rojo (MVZ 265696).

Appendix B. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2013.01.004.

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