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Trachemys in Mexico and beyond: Beautiful turtles, taxonomic nightmare, and a mitochondrial poltergeist (Testudines: Emydidae)

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Abstract

Trachemys is a speciose genus of freshwater turtles distributed from the Great Lakes in North America across the southeastern USA, Mexico and Central America to the Rio de la Plata in South America, with up to 13 continental American species and 11 additional subspecies. Another four species with three additional subspecies occur on the West Indies. In the present study, we examine all continental *Trachemys* taxa except for *Trachemys hartwegi* using mitochondrial and nuclear DNA sequences (3221 and 3396 bp, respectively) representing four mitochondrial genes and five nuclear loci. We also include representatives of all four West Indian species and discuss our results in the light of putative species-diagnostic traits in coloration and pattern. We provide evidence that one Mexican species, *T. nebulosa*, has captured a deeply divergent foreign mitochondrial genome that renders the mitochondrial phylogeny of *Trachemys* paraphyletic. Using nuclear markers, *Trachemys* including *T. nebulosa* represents a well-supported monophylum. Besides the mitochondrial lineage of *T. nebulosa*, there are six additional mitochondrial *Trachemys* lineages: (1) *T. venusta*, (2) *T. ornata* + *T. yaquia*, (3) *T. grayi*, (4) *T. dorbigni* + *T. medemi*, (5) *T. gaigeae* + *T. scripta*, and (6) West Indian *Trachemys*. These six mitochondrial lineages constitute a well-supported clade. Each mitochondrial *Trachemys* lineage is corroborated by our nuclear markers. For *T. gaigeae* another mitochondrial capture event is likely because its mitochondrial genome is sister to *T. scripta*, although *T. gaigeae* is deeply divergent in nuclear markers and resembles Mexican, Central and South American *Trachemys* species in morphology, sexual dimorphism and courtship behavior. The two subspecies of *T. nebulosa* and many Mexican and Central American subspecies of *T. venusta* are not clearly distinct in our studied genetic markers. Also, the putatively diagnostic coloration and pattern traits of the *T. venusta* subspecies are more variable than previously reported, challenging their validity. Our analyses fail to identify *T. taylori* as a lineage distinct from *T. venusta* and we propose to assign it as a subspecies to the latter species (*Trachemys venusta taylori* **nov. comb.**).

Keywords

Central America, integrative taxonomy, Mesoamerica, mitochondrial capture, museomics, North America, phylogeny, South America

† Deceased

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Introduction

Trachemys is a speciose and widely distributed genus of freshwater turtles (family Emydidae) occurring from the North American Great Lakes region through Central America to northern South America. Widely disjunct populations live in northeastern Brazil (Maranhão, Piauí) and in the Rio de la Plata region of Argentina, southern Brazil and Uruguay (TTWG 2021; Fig. 1). Thus, the genus ranges in north-south direction across a linear distance of more than 8200 km.

Continental America is home to up to 13 *Trachemys* species and 11 additional subspecies (TTWG 2021). Four further species with three additional subspecies occur on the West Indies. We follow Fritz et al. (2012, 2023) and recognize, besides the West Indian taxa, 11 continental *Trachemys* species with additional 11 subspecies (Table 1). *Trachemys* is a morphologically diverse genus, with taxa having a variegated ornamental pattern involving colorful ocelli, spots or streaks that are particularly obvious in hatchlings and juveniles. The maximum carapacial length of *Trachemys* ranges from 21 to 55 cm (straight line), with the largest taxa occurring in Central America (TTWG 2021). *Trachemys* are known as slider turtles, and their best-known representative is the red-eared slider (*T. scripta elegans*), a subspecies of *T. scripta*, widely distributed in the southcentral USA and adjacent Mexico. It has been introduced to many countries (TTWG 2021) and is listed among the 100 worst invasive species of the world (Lowe et al. 2004). Sliders, short for slider turtles, are omnivorous and occur in a variety of freshwater habitats, with a general preference for quiet waters with soft bodies, rich aquatic vegetation and suitable basking sites (Ernst and Barbour 1989). Males of some species display a complex innate courtship behavior that most likely acts as an isolating mechanism (Fritz et al. 1990). In some taxa the courtship behavior

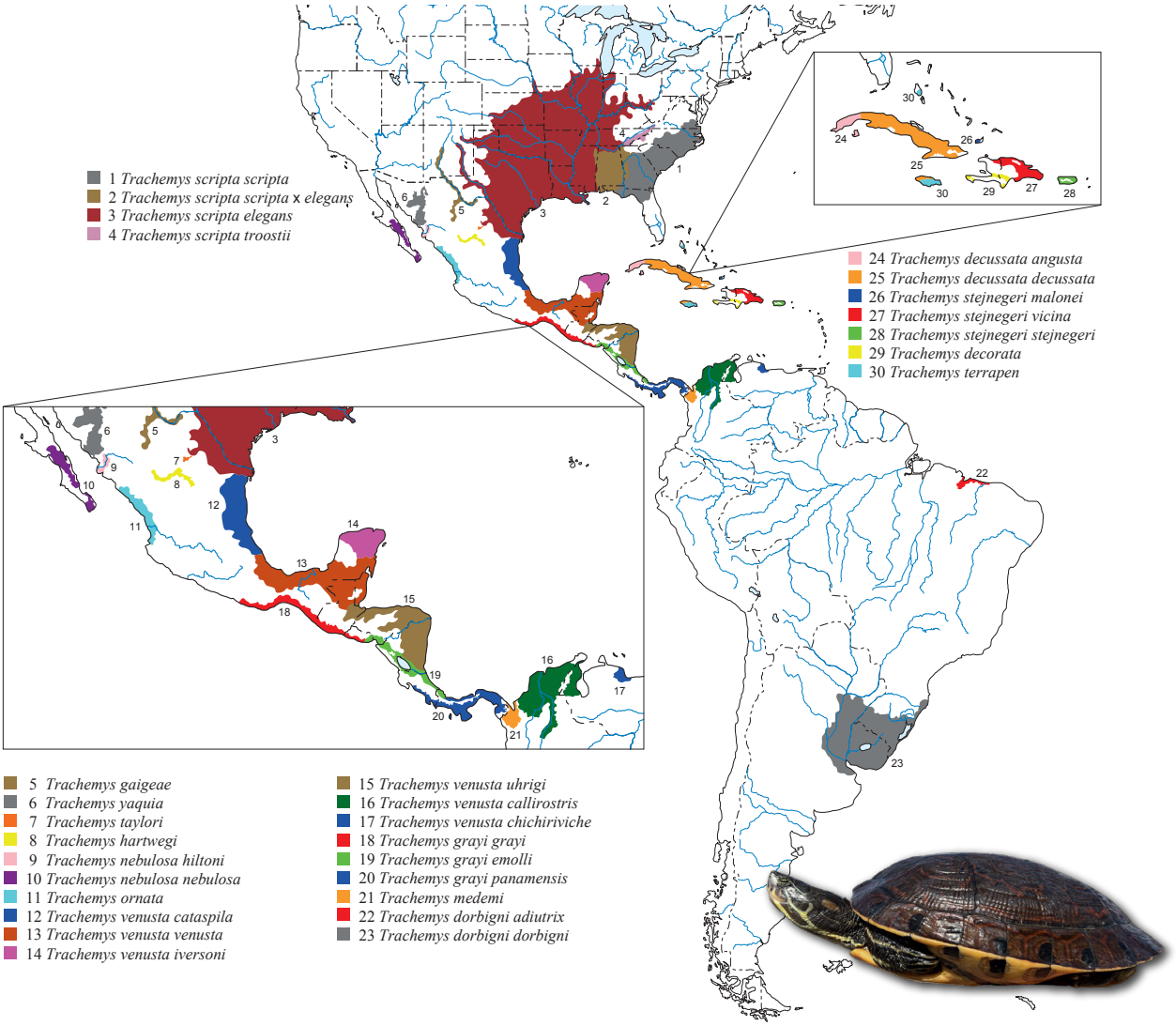

Figure 1. Distribution of *Trachemys* taxa (only putatively native occurrences). Map is compiled from species distribution maps in TTWG (2021), except for the ranges of *Trachemys grayi emolli* and *T. g. panamensis*. Populations south of Chiriquí Lagoon along the Caribbean coast of Panama are tentatively assigned to *T. g. panamensis* (see discussion in Fritz et al. 2023). Inset picture: *T. n. nebulosa* (photo: A. Monsiváis).

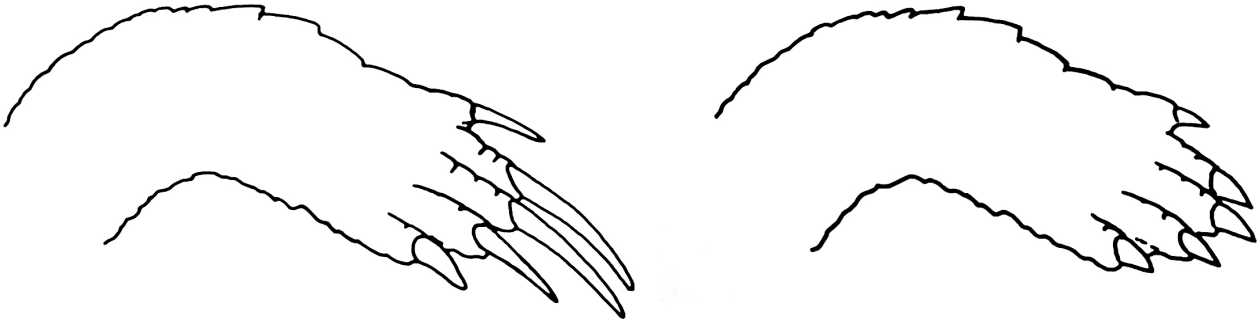

Figure 2. Elongated foreclaws of a male (left) *Trachemys scripta elegans* compared to a female (right). From Fritz (1990, redrawn from Cagle 1948).

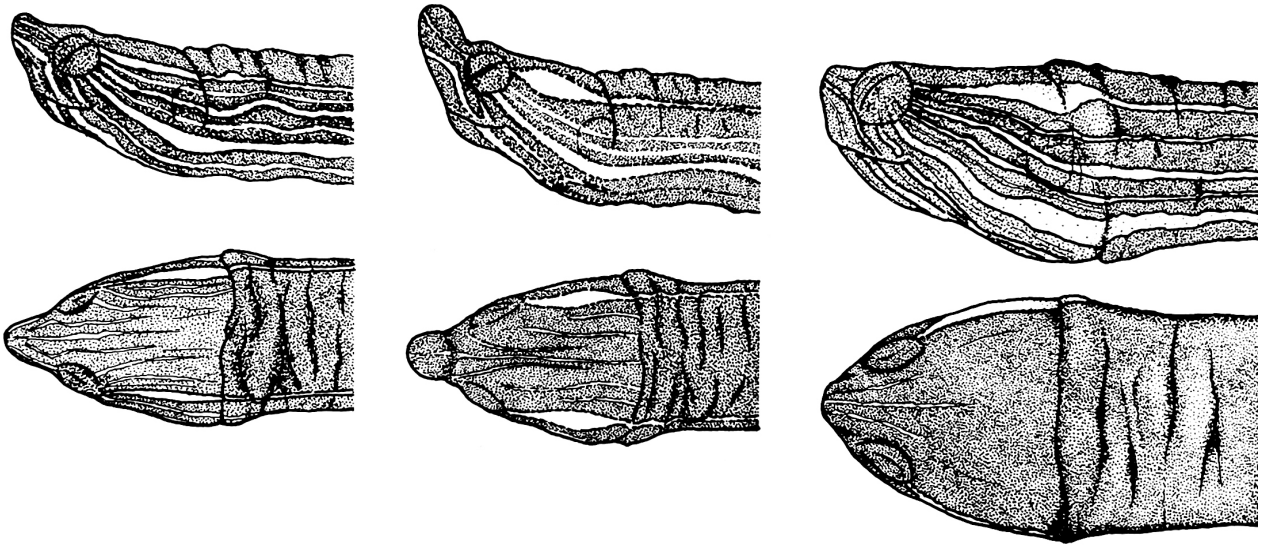

Figure 3. Sexual dimorphism in Central and South American slider turtles. Left, male *Trachemys grayi panamensis*, Juan Mina near Colón, Panama; center, male *T. v. venusta*, Tlacotalpan, Veracruz, Mexico; right, female *T. g. panamensis*, Juan Mina near Colón, Panama. Note the elongated and upturned snouts in the males. From Fritz (1990, turtles from Panama redrawn from Moll and Legler 1971).

involves trembling movements of the grotesquely elongated claws of the forefeet (Fig. 2) in front of the face of the female (*T. scripta*, Antillean species), the so-called "titillation behavior." In *T. gaigeae* and *T. venusta*, and probably in most Central and South American taxa, males instead perform vibrating or bobbing head movements in a vertical plane in front of the female's head. These head movements are emphasized sometimes by considerably elongated and upturned snouts (Fig. 3). However, neither of these behaviors have been described for the courtship of *T. taylori* (Ernst and Barbour 1989; Fritz 1990; Legler and Vogt 2013; Seidel and Ernst 2017).

For slider turtles, taxonomy is notoriously unstable. Both species delimitation and the number of recognized taxa have been contentious for decades (e.g., Moll and Legler 1971, Ernst 1990; Fritz 1990; Legler 1990; Seidel 2002; Fritz et al. 2012, 2023; Legler and Vogt 2013; Parham et al. 2015; see also the reviews in Seidel and Ernst 2017 and TTWG 2021). Legler and Vogt (2013) still advocated a single widely distributed polytypic species *T. scripta* ranging from southern Michigan, USA, to the Rio de la Plata region of temperate South America, in contrast to the up to 13 *Trachemys* species recognized by the Turtle Taxonomy Working Group (TTWG 2021).

Expanding previously published data from our lab (Fritz et al. 2012, 2023; Vargas-Ramírez et al. 2017; Vamberger et al. 2020), we focus here on the diversity of Mexican and Central American species and subspecies, where 15 taxa are thought to occur (Table 1). We included all but one of the 22 continental American species and subspecies and representatives of all four West Indian species in the present investigation. The only missing continental taxon is *T. hartwegi* from northern Mexico, which was originally described by Legler (1990) as a subspecies of *T. gaigeae*. As in our previous publications, we generate mitochondrial and nuclear DNA sequences to infer phylogeny and differentiation and place our results in a taxonomic and biogeographic context. As far as possible, we also use previously published information on external morphology, although the present study does not aim to provide an in-depth assessment of these traits and their taxonomic value. Based on these different lines of evidence, we develop the most complete taxonomic framework for *Trachemys* to date.

Table 1. *Trachemys* species recognized in the present study and their subspecies, with approximate distribution ranges from TTWG (2021) and Fritz et al. (2023; see there and Fig. 1 for more information). Genetic data were available for taxa bearing asterisks.

Taxon	Distribution
Trachemys decorata (Barbour & Carr, 1940)*	Hispaniola
Trachemys decussata (Bell, 1830)	
Trachemys decussata decussata (Bell, 1830)*	Cuba, Jamaica
Trachemys decussata angusta (Barbour & Carr, 1940)*	Cayman Islands, Cuba
Trachemys dorbigni (Duméril & Bibron, 1835)	
Trachemys dorbigni dorbigni (Duméril & Bibron, 1835)*	Argentina, southern Brazil, Uruguay
Trachemys dorbigni adiutrix Vanzolini, 1995*	Northern Brazil
Trachemys gaigeae (Hartweg, 1939)*	Northern Mexico, adjacent USA
Trachemys grayi (Bocourt, 1868)	
Trachemys grayi grayi (Bocourt, 1868)*	El Salvador, Guatemala, adjacent Mexico
Trachemys grayi emolli (Legler, 1990)*	Costa Rica, El Salvador, Honduras, Nicaragua, Panama
Trachemys grayi panamensis McCord, Joseph-Ouni & Blanck, 2010*	Costa Rica, Panama
Trachemys hartwegi (Legler, 1990)	Northern Mexico
Trachemys medemi Vargas-Ramírez, del Valle, Ceballos & Fritz, 2017*	Northern Colombia
Trachemys nebulosa (Van Denburgh, 1895)	
Trachemys nebulosa nebulosa (Van Denburgh, 1895)*	Northern Mexico
Trachemys nebulosa hiltoni (Carr, 1942)*	Northern Mexico
Trachemys ornata (Gray, 1830)*	Northern Mexico
Trachemys scripta (Schoepff, 1792)	
Trachemys scripta scripta (Schoepff, 1792)*	Southeastern USA
Trachemys scripta elegans (Wied-Neuwied, 1839)*	Southcentral USA, adjacent Mexico
Trachemys scripta troostii (Holbrook, 1836)*	Southeastern USA
Trachemys stejnegeri (Schmidt, 1928)	
Trachemys stejnegeri stejnegeri (Schmidt, 1928)	Puerto Rico
Trachemys stejnegeri malonei (Barbour & Carr, 1940)	Bahamas (Inagua)
Trachemys stejnegeri vicina (Barbour & Carr, 1940)*	Hispaniola
Trachemys taylori (Legler, 1960)*	Northern Mexico
Trachemys terrapen (Bonnaterre, 1789)*	Bahamas, Jamaica
Trachemys venusta	
Trachemys venusta venusta (Gray, 1856)*	Belize, Guatemala, southern Mexico
Trachemys venusta callirostris (Gray, 1856)*	Northern Colombia, Venezuela
Trachemys venusta chichiriviche (Pritchard & Trebbau, 1984)*	Venezuela
Trachemys venusta cataspila (Günther, 1885)*	Northern Mexico
Trachemys venusta iversoni McCord, Joseph-Ouni & Blanck, 2010*	Southern Mexico
Trachemys venusta uhrigi McCord, Joseph-Ouni & Blanck, 2010*	Guatemala, Honduras, Nicaragua
Trachemys yaquia (Legler & Webb, 1970)*	Northern Mexico

Materials and Methods

For 43 *Trachemys* samples (Table S1) the following mitochondrial genes were sequenced: 12S (partial), ND4L (complete), ND4 (complete), and cyt *b* (complete plus part of the adjacent tRNA-Thr gene). In addition, partial sequences of the nuclear loci Cmos (coding), ODC (exon 6, intron 6, exon 7, intron 7), R35 (intron 1), Rag1 (coding), and Rag2 (coding) were generated. Sequences from the present study are available under the European Nucleotide Archive (ENA) project accession number PRJEB75327; individual accession numbers are listed in Table S1. According to the state of preservation of the samples, we used different workflows.

Sanger sequencing. For 21 blood samples stored at –80°C as well as four additional samples of extracted DNA stored at –20°C we Sanger-sequenced the mentioned loci as described in Fritz et al. (2012) and Vargas-Ramírez et al. (2017). All reaction products were purified using SephadexTM G-50 fine (GE Healthcare). For selected samples including those of *T. n. nebulosa* and *T. n. hiltoni*, the long-range PCR approach described in Fritz et al. (2012) was used to corroborate that authentic mtDNA was sequenced.

Next Generation Sequencing (NGS) and in-solution hybridization capture. Eighteen further samples were taken from museum specimens (preserved between 1936 and 1996). Sequence data for this material were generated by an NGS approach including two rounds of in-solution hybridization capture. The historic material was processed in a cleanroom facility, physically isolated from the main laboratory, to avoid contamination by foreign DNA according to Fulton and Shapiro (2019). DNA ex-

traction was performed using the protocol by Patzold et al. (2020) with slight modifications (see Table S2) or Qiagen's DNeasy Blood & Tissue Kit according to the manufacturer's protocol, with a final elution of two times 50 µl elution buffer. Prior to the preparation of the Illumina sequencing libraries, DNA concentration and fragment length were assessed using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific) and a 4200 TapeStation system (Agilent). Where necessary, DNA was sheared down to an average length of 150 bp using a Covaris M220 ultrasonicator. Subsequently, DNA of four samples was converted into single-indexed, double-stranded DNA libraries (**dsLibs**) according to Meyer and Kircher (2010) with modifications by Fortes and Paijmans (2015). Due to a possible preservation with formalin, the remaining 14 historic samples were converted into double-indexed, single-stranded DNA libraries (**ssLibs**) according to Gansauge and Meyer (2019). In order to increase the amount of the targeted loci in all DNA libraries, two-rounds of in-solution hybridization capture (Maricic et al. 2010; Horn 2012) were performed in a dedicated capture-only workspace in the main laboratory using DNA baits generated from modern PCR products. For the mtDNA bait library, two long-range PCR reactions were performed (LR1 and LR2) using a sample of *Trachemys venusta callirostris* (MTD T 4728), yielding amplicons with an overlap of 1136 bp and an individual length of 11,760 bp (LR1) and 6686 bp (LR2). The combined long-range PCR products covered most of the mitochondrial genome (mitogenome) from tRNA-Phe (situated before 12S) to the 3'-end of the control region, missing out approximately 200 bp. By aiming at large stretches of mtDNA, the risk of amplifying nuclear copies of mitochondrial DNA (numts), which are an issue in *Trachemys*, is minimized (Bensasson et al. 2001; Fritz et al. 2012). For each long-range PCR, a 50 µl volume was used, containing 1 unit of TaKaRa LA Taq DNA Polymerase, Hot-Start Version (Clontech Laboratories Inc.), and the reaction mixture recommended by the manufacturer. PCR conditions comprised initial denaturation at 93°C for 3 min, followed by 35 cycles of 93°C for 20 sec, 57°C for 30 sec, 68°C for 12 min, and a final elongation step at 68°C for 20 min; for primer sequences see Table S3. PCR products were visualized and excised from a 2% agarose gel and purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel). After pooling both long-range products at an equimolar rate, the baits were sheared down to 150 bp and converted into the mtDNA bait library. The nDNA bait library was produced from PCR products of *T. v. callirostris* (MTD T 4728) and *T. scripta elegans* (MTD T 12680) obtained as detailed in Fritz et al. (2012) and Praschag et al. (2017); primer sequences are given in Table S3. The PCR products for the five nuclear loci were pooled at an equimolar rate, sheared down to 150 bp and converted into the nDNA bait library. Prior to capturing, the mtDNA and nDNA bait libraries were adjusted to the same molarity and mixed at a ratio of 1:5 (for dsLibs) or 1:10 (for ssLibs) to account for the lower numbers of nuclear target molecules in the DNA libraries of the individual samples. Sequencing was performed in-house on an Illumina MiSeq platform, generating 75 bp-long paired-end reads.

Bioinformatics. NGS sequence data were assembled using the following pipeline: After adapter trimming with Skewer 0.2.2 (Jiang et al. 2014), read merging (minimum length 35 bp), quality filtering (minimum Q-score 20), and duplicate removal with BBmap-suite 37.24 (https://sourceforge.net/projects/bbmap) (Bushnell et al. 2017), the remaining reads (**readpool-0**) were screened for contamination using FastQScreen 0.11.4 (Wingett and Andrews 2018) and a set of predefined mitochondrial and nuclear sequences (Table S4), including the mitogenome of *T. s. elegans* (GenBank accession number KM216748) and a concatenated sequence of the five nuclear loci of one specimen of *T. medemi* (LT883198, LT883245, LT883260, LT883222, LT883234)—the individual loci being separated by stretches of 2000 ambiguous sites (Ns) to prevent mapping artifacts. The identified non-*Trachemys* reads were excluded from the individual readpools, and the remaining reads were stored as **readpool-1**. Reads only mapping to the mitogenome of *T. s. elegans* were stored as **readpool-2**. Near-complete mitogenomes were assembled using MITObim (Hahn et al. 2013), a two-step baiting and iterative mapping approach, readpool-1 (ssLibs) or readpool-2 (dsLibs), an allowed mismatch value of 2, and sequence KM216748 (*T. s. elegans*) as a starting seed. For step 1 of the mapping procedure of the dsLibs, readpool-2 was reduced to 30,000 randomly selected reads. The five nuclear loci of the 14 ssLibs were also assembled with MITObim using individual mapping events, readpool-0, an allowed mismatch value of 2, and the above-mentioned sequences of *T. medemi* as a starting seed. The nuclear assemblies of the four dsLibs were carried out in a single mapping event per sample, using the Burrows-Wheeler Aligner (Li and Durbin 2009) with its Maximal Exact Match algorithm (BWA-MEM), applying a relaxed mismatch threshold of 0.001 (corresponding to approximately eight mismatches in 100 bp), readpool-1 including all non-merged quality-filtered reads due to the low number of available merged reads, and the concatenated nuclear sequences of *T. medemi* as a mapping reference. All resulting scaffolds were visualized and checked for assembly artifacts in Tablet (Milne et al. 2013). Assembly artifacts were manually removed from the assembled contigs and all positions with a coverage below 3-fold masked as ambiguous (N), using the maskfasta subcommand of BEDTools 2.29.2 (Quinlan and Hall 2010). Sequence length distribution of mapped reads was calculated with a customized awk command and Microsoft Excel. The temporal authenticity of the mapped reads was tested with mapDamage 2.0 (Jónsson et al. 2013), which accounts for nucleotide misincorporations due to DNA degradation. An exemplary sample documentation for the oldest specimen processed in this study (SMF 22291, collected 1936) is provided in Figures S1–S6. The summarized mapping details are provided in Table S5.

Alignment preparations. The new Sanger-sequenced data were visually inspected for base-calling errors and

then aligned with the NGS data and previously published sequences of 90 *Trachemys* and related taxa (Table S1). Eight individual files (12S, ND4L/ND4, cyt *b* plus tRNA-Thr, Cmos, ODC, R35, Rag1, Rag2) were created using BioEdit 7.0.5.2 (Hall 1999). NGS sequence data were added by extracting the appropriate genes from the mitochondrial and nuclear assemblies. The individual alignments were adjusted manually and cropped to their final lengths. Each protein-coding gene was screened for the presence of internal stop codons using MEGA X (Kumar et al. 2018). For the three mitochondrial alignments, problematic sequence features were removed manually before the sequences were concatenated for analysis (4 bp of stop codons of coding genes as these do not code for any amino acid; 11 bp of gene overlap including adjacent codon positions to maintain an intact reading frame because these short regions cannot be identified with a single gene and may have evolved differently; 3 bp of intergenic spacer regions). The mtDNA alignment used for calculations was 3221 bp long and contained sequences from 133 turtles (12S: 398 bp; cyt *b*: 1137 bp; tRNA-Thr: 26 bp; ND4L: 289 bp; ND4: 1371 bp).

The coding regions of the five nuclear loci were also checked for internal stop codons before being concatenated. After obtaining both alleles for each sample by phasing each sequence with DnaSP 6.12 (Rozas et al. 2017), the final alignment had a length of 3396 bp (Cmos: 563 bp; ODC: 620 bp; R35: 962 bp; Rag1: 614 bp; Rag2: 637 bp) and contained 212 phased sequences from 106 specimens.

Phylogenetic analyses. Phylogenetic analyses were performed for the mitochondrial and nuclear datasets independently, applying Maximum Likelihood (ML) and Bayesian Inference (BI) approaches using RAxML 8.0.0 (Stamatakis 2014) and MrBayes 3.2.6 (Ronquist et al. 2012). The best evolutionary models and partitioning schemes (Tables S6–S9) were determined with PartitionFinder2 (Lanfear et al. 2016) applying the greedy search scheme and the Bayesian Information Criterion. For ML, 10 independent searches were carried out using the GTR + G substitution model, different starting conditions, and the rapid bootstrap option, following the recommendation of Stamatakis (2016: 59, 60) to avoid the GTR+I+G model. Subsequently, 1000 non-parametric thorough bootstrap replicates were calculated and the values plotted against the best tree. For BI, four parallel runs (each with eight chains) were performed with 5 million (mitochondrial dataset) and 10 million (nuclear dataset) generations (burn-in 0.25; print frequency 1000; sample frequency 1000). Calculation parameters were analyzed using Tracer 1.7.1 (Rambaut et al. 2018).

SplitsTree analysis. In addition, the phased and concatenated nuclear DNA dataset was used to build a phylogenetic network in the program SplitsTree4 v4.18.3 (Bryant and Huson 2023) based on uncorrected p distances and the NeighborNet algorithm. The appropriate alignment was reduced to 82 specimens (164 sequences), containing only those samples with all five nuclear loci present and a maximum of 5% missing data.

Mitochondrial molecular clock. To estimate the approximate time of mitochondrial capture in *T. nebulosa* and *T. gaigeae* (see below), we run exploratory calculations using the uncorrelated relaxed molecular clock implemented in BEAST 1.8.2 (Drummond and Rambaut 2007) and a partitioned dataset of concatenated mtDNA sequences. The alignment included only one representative for each taxon, except for *T. v. venusta* for which two divergent sequences were included. The same settings and fossil calibrations as in Fritz et al. (2012) were employed using lognormal prior distributions for the most recent common ancestors. To achieve effective sample sizes (ESS) for all parameters that exceeded 100, the analyses were run for 350 million generations.

Results

Mitochondrial phylogeny

The two tree-building approaches delivered similar results (Figs 4, 5). In agreement with earlier studies (Fritz et al. 2012, 2023; Vargas-Ramírez et al. 2017), the previously studied taxa correspond to five deeply divergent and well-supported clades within *Trachemys*. These are, in Figure 5 from top to bottom, one clade each for the two Mexican and Central American species (1) *T. venusta*, a species having also two subspecies in northern South America (*T. v. callirostris*, *T. v. chichiriviche*), and (2) *T. grayi*, (3) a further clade comprising the South American species *T. dorbigni* + *T. medemi*, (4) another clade consisting of the North American species *T. scripta* + *T. gaigeae*, and (5) yet another clade containing all West Indian species (*T. decorata*, *T. decussata*, *T. stejnegeri*, *T. terrapen*). However, there are two additional clades, one comprised of sequences of *T. yaquia* and *T. ornata* and the other clade, of the two subspecies of *T. nebulosa* (see below).

Within *T. grayi*, the three currently recognized subspecies *T. g. grayi*, *T. g. emolli* and *T. g. panamensis* and within *T. dorbigni*, the two subspecies *T. d. dorbigni* and *T. d. adiutrix* represent reciprocally monophyletic clades. However, this is not the case with respect to the subspecies of *T. venusta*. Also, the placement of some previously unstudied taxa was unexpected and two taxa are not distinct. Our only representative of *T. v. iversoni* clusters within *T. venusta* and shares with some *T. v. venusta* and *T. v. uhrigi* the same mitochondrial lineage. *Trachemys taylori* clusters within *T. venusta* as well. In contrast, sequences of *T. yaquia* and *T. ornata* are distinct and reciprocally monophyletic. They represent together a well-supported and deeply divergent clade which is, with high support, sister to *T. venusta*.

The clade (*Trachemys yaquia* + *T. ornata*) + *T. venusta* is with weak support sister to *T. grayi*. These four mainly Mexican and Central American species represent together a well-supported clade that also contains the South American taxa *T. dorbigni* + *T. medemi*. This

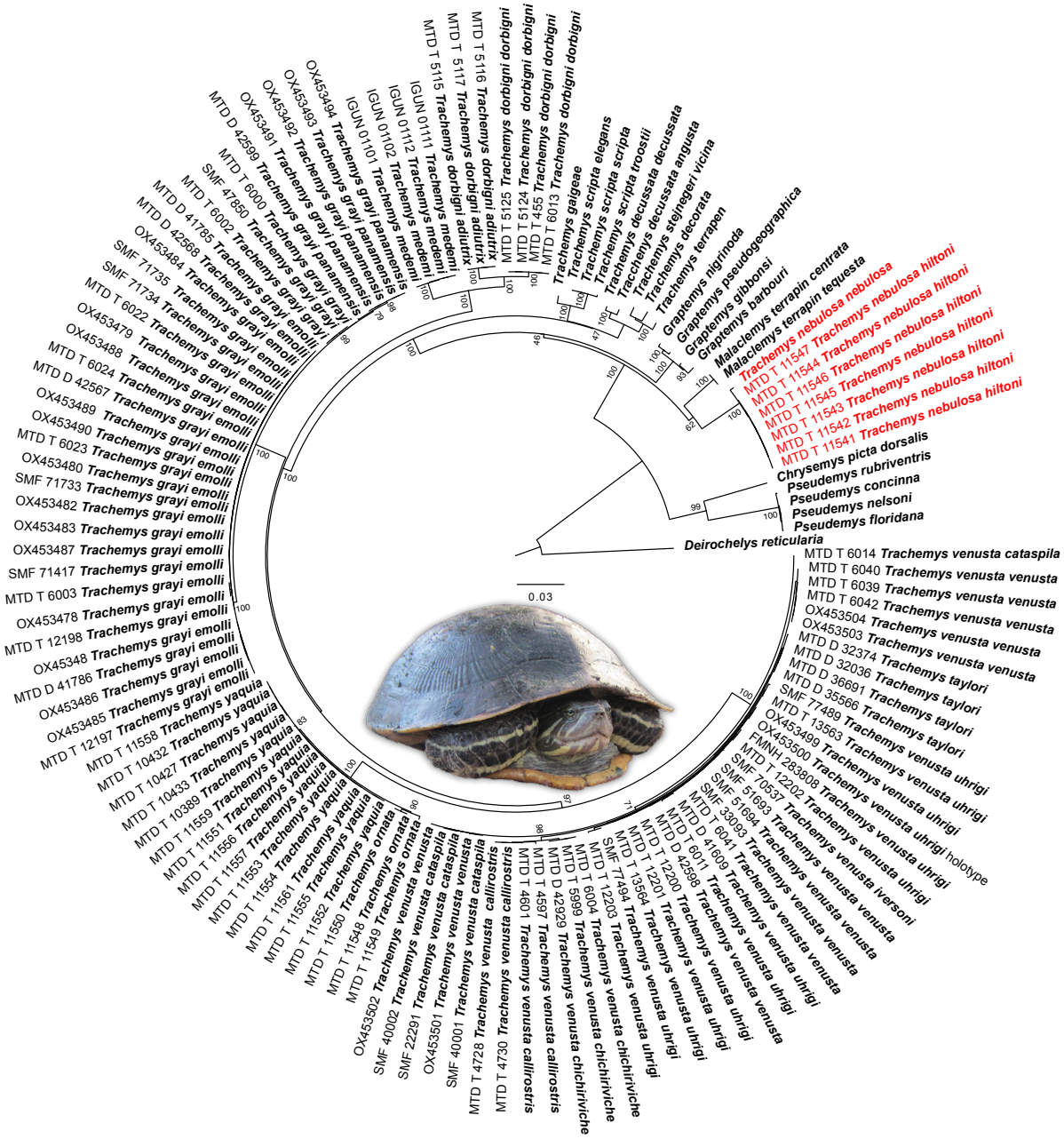

Figure 4. Mitochondrial phylogeny of *Trachemys* species and related taxa as inferred by RAxML 8.0.0, rooted with *Deirochelys reticularia* and based on 3221 bp of mtDNA (12S, ND4L, ND4, cyt *b* plus adjacent tRNA-Thr, 133 specimens). Codes preceding taxon names are voucher or ENA accession numbers (see also Table S1). Numbers at nodes are bootstrap values. Note the placement of *Trachemys nebulosa* (red) outside *Trachemys* as sister lineage of *Malaclemys terrapin* and the well-supported monophyly of the remaining *Trachemys* taxa. Inset picture: *T. n. hiltoni* (photo: P. C. Rosen).

more inclusive clade comprised of Mexican, Central and South American taxa occurs in an unresolved but well-supported clade that also contains the two clades of North American and West Indian *Trachemys*. Notably, *T. nebulosa* is excluded from this *Trachemys* clade and appears unexpectedly with weak support as sister taxon of the diamondback terrapin *Malaclemys terrapin*. This latter *Malaclemys* + *T. nebulosa* clade and another clade corresponding to *Graptemys* occur together with the monophyletic *Trachemys* exclusive *T. nebulosa* in an unresolved polytomy in another well-supported clade; the sister group of this clade is *Chrysemys* + *Pseudemys*.

Our only sequence of the nominotypical subspecies of *T. nebulosa* from the Baja California Peninsula is not clearly differentiated from seven *T. n. hiltoni* from Sinaloa.

Mitochondrial divergence within *Trachemys* commenced 6.1 million years ago (mya; Fig. S7). The mitochondrial divergence between *T. gaigeae* and *T. scripta* was dated to 2.2 mya; and that between *T. nebulosa* and *M. terrapin*, to 7.0 mya. All obtained estimates were younger than those presented in Fritz et al. (2012) for a similar taxon sampling (Table S10). However, the calculations in Fritz et al. (2012) included in addition to the same mitochondrial genes also the five nuclear loci of the present study.

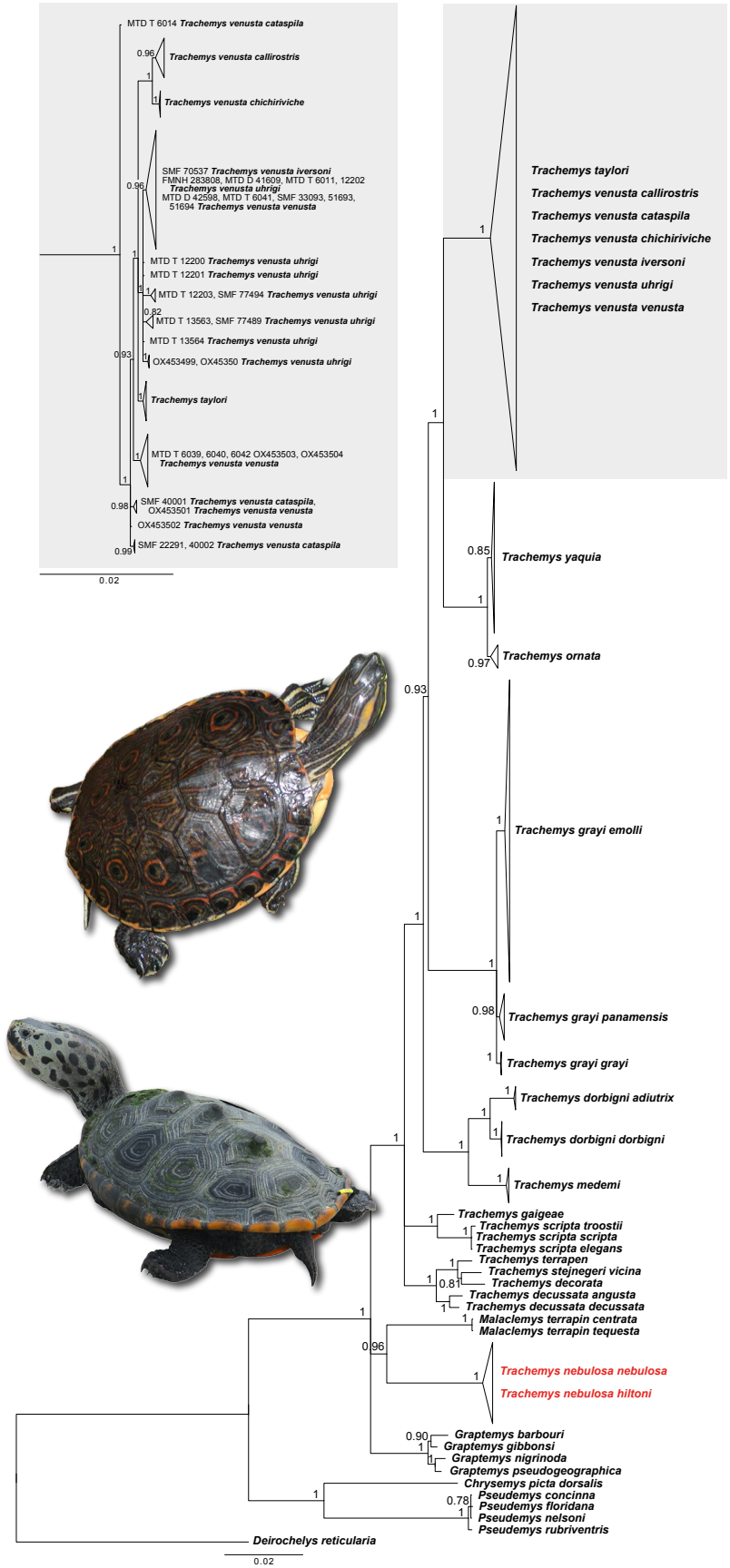

Figure 5. Mitochondrial phylogeny of *Trachemys* species and related taxa as inferred by MrBayes 3.2.6, rooted with *Deirochelys reticularia* and based on 3221 bp of mtDNA (12S, ND4L, ND4, cyt *b* plus adjacent tRNA-Thr, 133 specimens). Clades collapsed to cartoons. Codes preceding taxon names are voucher or ENA accession numbers (see also Table S1). Numbers at nodes are posterior probabilities. Note the placement of *Trachemys nebulosa* (red) outside *Trachemys* as sister lineage of *Malaclemys terrapin* and the well-supported monophyly of the remaining *Trachemys* taxa. Grey rectangle top left shows details for right grey rectangle. Inset pictures, top and bottom: *T. v. venusta* (photo: U. Fritz) and *M. terrapin* (photo: A. T. Coleman).

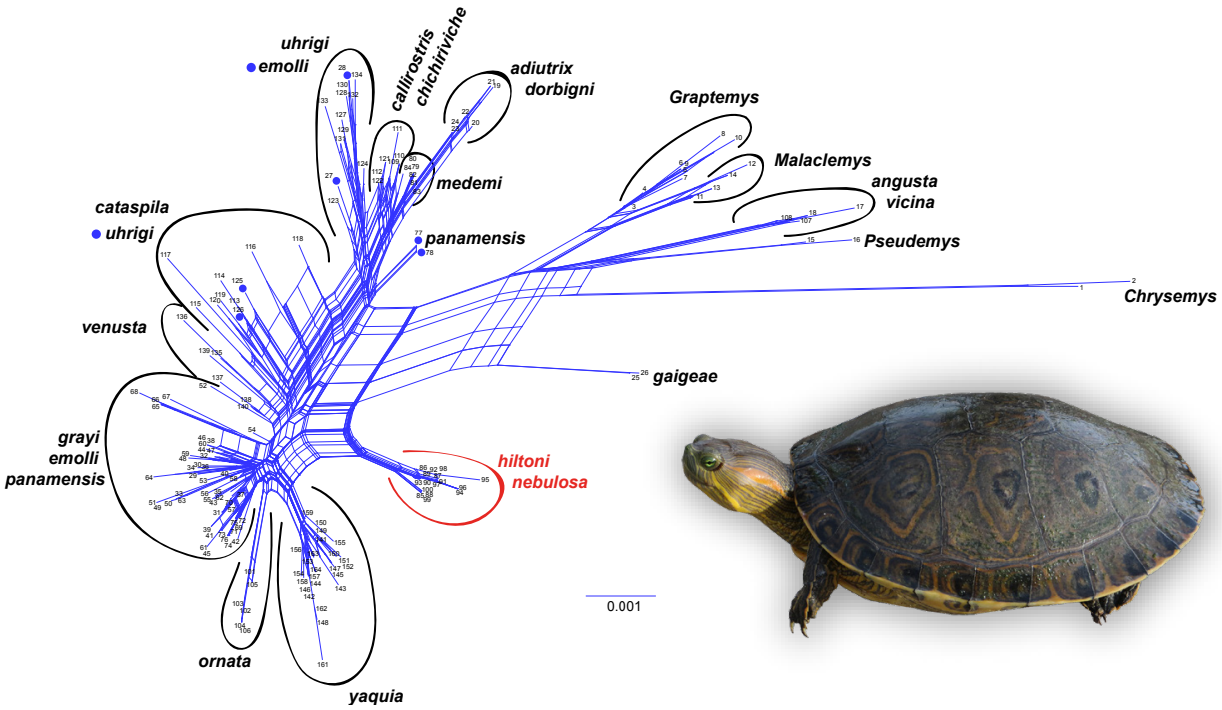

Figure 6. SplitsTree for phased and concatenated nuclear DNA sequences of *Trachemys* species and related taxa (Cmos, ODC, R35, Rag1, Rag2, 3396 bp, 82 specimens; sequences with less than 5% missing data). Numbers at branch tips refer to alleles, see Table S1 for explanation. Note the similarity of *Graptemys* and *Malaclemys* and the placement of *Trachemys nebulosa* (red) next to the geographically neighboring *Trachemys* taxa (*T. gaigeae*, *T. ornata*, *T. yaquia*). Conflicting samples highlighted with solid blue circles. Inset picture: *T. ornata* (photo: P. C. Rosen).

Nuclear phylogeny

Using our five nuclear loci Cmos, ODC, R35, Rag1, and Rag2, the relationships of the studied taxa are only incompletely resolved. However, several firm conclusions can be deduced.

Our SplitsTree analysis using a phased dataset with a maximum of 5% missing sequence data (Fig. 6) clearly places *T. nebulosa* into the same cluster as all other Mexican and Central American taxa; *Graptemys* and *Malaclemys* are closely related and highly divergent. There is no evidence for a close relationship of *Malaclemys* and *T. nebulosa*. *Trachemys gaigeae*, the Antillean taxa *T. decussata angusta* and *T. stejnegeri vicina*, *Pseudemys* and *Chrysemys* represent further deeply divergent branches or clusters. Within the cluster comprised of Central and South American *Trachemys*, most species were returned as distinct subclusters or distinct branches. Conversely, most subspecies could not be resolved, even though *T. v. venusta*, *T. v. cataspila* and *T. v. uhrigi* appear as largely distinct subclusters. However, their distinctness is not supported in the phylogenetic analyses (Figs S8, S9, see below). The two South American subspecies *T. v. callirostris* and *T. v. chichiriviche* represent together another weakly distinct subcluster in the SplitsTree analysis (Fig. 6), with an intermediate position between the subcluster of *T. v. uhrigi* and the South American species *T. medemi* and *T. dorbigni*. Though, in the phylogenetic analyses, the placement of *T. v. callirostris* and *T. v. chichiriviche* is unresolved and without statistical support (Figs S8, S9). The single specimen of *T. n. nebulosa* was included in the same SplitsTree subcluster as seven *T. n. hiltoni* (Fig. 6). With regard to species allocation, two *T. grayi* samples were misplaced. One *T. g. panamensis* (MTD D 42599, allele numbers 77 and 78 in Fig. 6) represents a distinct cluster and one *T. g. emolli* (SMF 71417, numbers 27 and 28 in Fig. 6) was placed among *T. v. uhrigi*. The alleles of one *T. v. uhrigi* clustered among *T. v. cataspila* (FMNH 283808, numbers 125 nd 126 in Fig. 6).

RAxML and MrBayes analyses using all phased sequences (Figs S8, S9), i.e., also those with more than 5% missing data, confirm the general patterns. Notably, the two algorithms place *T. nebulosa* into a well-supported clade together with the other Mexican, Central and South American *Trachemys* species, i.e., *T. dorbigni*, *T. grayi*, *T. medemi*, *T. ornata*, *T. venusta* (including *T. taylori*), and *T. yaquia*. Furthermore, *Graptemys* and *Malaclemys* are deeply divergent and well-supported sister taxa. The two museum specimens of *T. taylori* for which nuclear DNA sequences could be obtained, are placed within *T. venusta* and not distinct from sequences from *T. v. cataspila*. These two specimens were not included in the SplitsTree calculation due to missing data. The sequences of the two subspecies of *T. nebulosa* were slightly distinct in the trees, in contrast to the SplitsTree analysis. The unexpected position of the two above-mentioned *T. grayi* samples (MTD D 42599, SMF 71417) and the *T. v. uhrigi* (FMNH 283808) is also reflected in the phylogenetic trees.

Even though the trees are generally not well resolved, the following additional observations are noteworthy (i) the South American species *T. dorbigni* (with the subspecies *T. d. dorbigni* and *T. d. adiutrix*) and *T. medemi*

are well-supported sister taxa within the Mexican, Central and South American clade; (ii) sequences of the two *T. nebulosa* subspecies are another well-supported subclade within the Mexican, Central and South American clade and the only representative of *T. n. nebulosa* is distinct from *T. n. hiltoni*; (iii) *T. grayi*, *T. taylori* and *T. venusta* are not reciprocally monophyletic; (iv) sequences of *T. taylori* cluster with sequences of *T. v. cataspila*; (v) several sequences of *T. v. uhrigi* represent a weakly supported subclade that corresponds to the subcluster for *T. v. uhrigi* in the SplitsTree; this subclade includes 11 (MrBayes) or 13 (RAxML) sequences of *T. v. uhrigi* from Guatemala, Honduras, Nicaragua and the two alleles of one *T. g. emolli* (SMF 71417) from Costa Rica, (vi) the remaining 13 or 11 of the 24 sequences of *T. v. uhrigi* (which were mostly not used in the SplitsTree because of missing data) appear in remote positions across the Mexican, Central and South American clade, either in unresolved polytomies or they cluster with weak support with alleles of *T. v. venusta*, *T. v. cataspila*, *T. v. chichiriviche*, or *T. g. panamensis*; (vii) *T. ornata* and *T. yaquia* constitute distinct subclades within the Mexican, Central and South American clade; (viii) the only representative of *T. gaigeae* is deeply divergent from the Mexican, Central and South American clade and clusters within an unresolved polytomy that also contains the other North American taxa, i.e., *T. scripta*, a well-supported clade comprised of *Malaclemys* with *Graptemys* as its the well-supported sister, *Chrysemys* and *Pseudemys*, and the West Indian *Trachemys* taxa; (ix) within this polytomy, the West Indian species represent a well-supported monophylum; (x) some sequences of the North American *T. scripta* are sister to the West Indian taxa, while others cluster with weak support with *Pseudemys*.

A long discussion, with external morphology and courtship behavior not making things easier

The results of our analyses based on mitochondrial and nuclear DNA are not in complete agreement. When the mitochondrial and nuclear topologies are compared (Figs 4, 5, 6, S8, S9), the most notable difference is the conflicting placement of *Trachemys nebulosa*. In the mitochondrial phylogenies (Figs 4, 5), *T. nebulosa* is with weak support sister to *Malaclemys terrapin*, a highly distinctive species confined to coastal zones with brackish water. It is distributed farther east along a narrow strip following the southern and eastern US coasts from Texas to Cape Cod and represents a monotypic genus (Ernst and Barbour 1989; Seidel and Ernst 2017; TTWG 2021). In sharp contrast, our analyses based on nuclear DNA (Figs 6, S8, S9) reveal that *T. nebulosa* belongs to the Mexican and Central American *Trachemys* clade, in agreement with its traditional taxonomic assignment (e.g., Moll and Legler 1971; Ernst and Barbour 1989; Legler 1990; Seidel and Ernst 2017; TTWG 2021). According to the nuclear data, *Malaclemys* is phylogenetically deeply divergent from *Trachemys* and sister to the genus *Graptemys*, the morphologically distinctive map and sawback turtles from southeastern North America. This topology is in line with previous investigations (see the reviews in Seidel and Ernst 2017 and TTGW 2021) including a study based on 15 nuclear genes (Thomson et al. 2021). However, the sister group relationship of *T. nebulosa* + (*T. dorbigni* + *T. taylori*) shown in the tree in Thomson et al. (2021) is questionable because samples misidentified on the species level seem to have been used for *Trachemys* (Fritz et al. 2023). Further insecurity arises from the conflicting placement of *T. nebulosa* in the tree presented in the supplementary information of Thomson et al. (2021), where *T. nebulosa* is sister to a clade comprised of all other Mexican, Central and South American *Trachemys* taxa.

Already the results of the pioneering study by Wiens et al. (2010) on the relationships of emydid turtles resembled our mitochondrial topologies in that a single sample of *T. nebulosa* was with weak support sister to *Malaclemys* in their tree based on mitochondrial cyt *b* and ND4 sequences, while it was the well-supported sister taxon of a single sample of *T. venusta* in an analysis using six nuclear loci. Seidel and Ernst (2017) suggested that the mitochondrial results of Wiens et al. (2010) were erroneous, which seemed reasonable in the light of the phylogeny presented by Parham et al. (2015). The latter authors included in their analysis of Central American and Mexican *Trachemys* both *T. n. nebulosa* and *T. n. hiltoni*, which were sister taxa and occurred on a long branch within a monophyletic *Trachemys*. Parham et al. (2015) used a combined dataset of mitochondrial (ND4) and nuclear (R35) DNA sequences. Therefore, the placement of the two *T. nebulosa* subspecies was due to the signal of the nuclear locus. A comparison of the mtDNA sequences from Wiens et al. (2010) and Parham et al. (2015) with ours shows a complete match.

Mitochondrial phylogenies of *Trachemys* and related emydids can be easily confounded by the unintended inclusion of numts (non-coding nuclear mitochondrial DNA insertions) obtained with standard PCR primers (see Fritz et al. 2012). However, this is unlikely for *T. nebulosa*. We used for all of our Sanger-sequenced samples the approach described in Fritz et al. (2012) to minimize the risk of amplifying numts. This approach involves long-range PCR and tailored primers. Thus, we are confident that our mitochondrial DNA sequences are authentic. However, how can the conflicting evidence for nuclear and mitochondrial DNA be explained?

Mitochondrial introgression and mitochondrial capture are known to have occurred in multiple turtle clades and sometimes across deeply divergent taxa (Chelidae: *Chelodina*, *Emydura*, *Myuchelys* – Hodges 2015; Kehlmaier et al. 2019, 2024; Emydidae: *Actinemys*, *Graptemys* – Spinks and Shaffer 2009; Praschag et al. 2017; Geoemydidae: *Cuora*, *Cyclemys*, *Malayemys*, *Rhinoclemmys* – Spinks and Shaffer 2007; Fritz et al. 2008; Vargas-Ramírez et al. 2013; Ihlow et al. 2016; Vamberger et al. 2017; Kinosternidae: *Kinosternon* – Hurtado-Gómez et al. 2024; Trionychidae: *Pelodiscus* – Gong et al. 2018). Fritz et al. (2023) also considered mitochondrial capture for *T. gaigeae*, whose mitogenome is the sister lineage

of North American *T. scripta*, even though *T. gaigeae* is highly distinct according to nuclear information (see also our Figs 6, S8, S9) and resembles Mexican, Central and South American *Trachemys* taxa in morphology, sexual dimorphism and the head bobbing behavior during courtship (Legler and Vogt 2013).

This situation suggests that the deeply divergent mitochondrial lineage in *T. nebulosa* represents another case of mitochondrial capture, either from the ancestor of the extant *Malaclemys terrapin* or its extinct sister taxon. According to our exploratory molecular clock calculations, the mitochondrial lineages of *M. terrapin* and *T. nebulosa* diverged 7.0 mya (95% HPD: 5.2–9.6 mya; Fig. S7 and Table S10), and this estimate might reflect the approximate time of mitochondrial capture. It is remarkable that this estimate predates that for the divergence of the mitochondrial lineages within *Trachemys*, even though the 95% HPD intervals widely overlap (5.2–9.6 mya and 4.9–7.7 mya; Table S10). However, there are several caveats. In particular, the inferred date could correspond to the divergence of the ancestor of *M. terrapin* and its extinct sister taxon, and not to the date of the mitochondrial capture, i.e., *T. nebulosa* could have captured the mitogenome later. Furthermore, the calibration points used may be misleading because the divergence history of mitogenomes is not necessarily congruent with the diversification of the 'host' organisms. Mitochondrial genes behave like a single locus, and a molecular clock should be ideally applied to a species tree or a multilocus dataset, not a single locus. Also, the mitochondrial sister group relationship of *T. nebulosa* and *M. terrapin* is only weakly supported and the foreign mitogenome of *T. nebulosa* could originate from another extinct emydid lineage. In any case, the divergence time estimate and the deep divergence of the mtDNA of *T. nebulosa* suggest that the mitochondrial capture occurred very early during the diversification of *Trachemys*, perhaps when the diversification of the genus began. In contrast, *T. gaigeae* captured its mitogenome from the ancestor of *T. scripta* much later, although our estimate of 2.2 mya (Table S10) should be treated with the same reservations as for *T. nebulosa*.

Our nuclear dataset of five loci obviously does not completely resolve the phylogeny of *Trachemys*. However, the placement of *T. nebulosa* is consistent in SplitsTree, Bayesian and Maximum Likelihood analyses (Figs 6, S8, S9) and well supported. In addition, and in agreement with the calculations presented in Fritz et al. (2023), the analyses of our nuclear data allow further insights. Mexican, Central and South American *Trachemys* taxa appear to be closely related, while *T. scripta*, the West Indian taxa and *T. gaigeae* are distinct. The placement of some taxa and sequences in our SplitsTree analysis makes geographic sense, suggestive of past or current gene flow. This is true for the clusters of the South American taxa *T. d. dorbigni*, *T. d. adiutrix* and *T. medemi* which are neighbors of the geographically close South American subspecies of *T. venusta* (*T. v. callirostris*, *T. v. chichiriviche*; Fig. 6). Furthermore, five individuals of the southern Central American subspecies *T. v. uhrigi* are neighbor to the South American taxa. Also, the geographically close *T. ornata* and *T. yaquia* are neighbors in the SplitsTree. However, neither *T. v. venusta* and *T. v. cataspila* nor the three subspecies of *T. grayi* (*T. g. grayi*, *T. g. emolli*, *T. g. panamensis*) are clearly distinct.

Based on a cladistic analysis of morphological traits, Seidel (2002) recognized the allopatric taxa *T. v. callirostris* and *T. v. chichiriviche* as subspecies of a distinct species, *T. callirostris*. However, his results are questionable in the light of current genetic evidence. They included many untenable findings, such as the deeply divergent non-sister placement of two populations of *T. d. dorbigni* ('*brasiliensis*' and '*dorbigni*'), or the non-monophyly of the Antillean taxa and of *T. grayi*, or the paraphyly of *T. venusta* with respect to *T. g. grayi* (Seidel 2002: fig. 2). According to gross morphology, *T. v. callirostris* and *T. v. chichiriviche* differ little from other *T. venusta* subspecies. With respect to mtDNA, *T. v. callirostris* and *T. v. chichiriviche* are embedded in the remaining mitochondrial lineages of *T. venusta* (Figs 4, 5), and our nuclear markers do not provide unambiguous evidence for any classification (Figs 6, S8, S9). We therefore continue to treat *T. v. callirostris* and *T. v. chichiriviche* as subspecies of *T. venusta*, as proposed in Fritz et al. (2012).

Some sequences in the SplitsTree analysis are at first glance misplaced (highlighted with blue circles in Fig. 6). This could reflect ancestral polymorphism or hybridization. The latter option seems likely for a specimen (SMF 71417) morphologically identified as *T. g. emolli* (in agreement with its mitochondrial identity; see Figs 4, 5), which clusters within *T. v. uhrigi*. It originates from Costa Rica, where a contact zone of *T. g. emolli* and *T. v. uhrigi* along the Caribbean zone is expected (see Fritz et al. 2023). Regarding another misplaced specimen (MTD D 42599, *T. g. panamensis*) from Panama, Fritz et al. (2023) speculated about an artifact because all mutations separating this specimen from other *T. g. panamensis* occur in only one nuclear locus. However, hybridization and subsequent recombination could also explain this. A third misplaced specimen is a *T. v. uhrigi* from Honduras (FMNH 283808), which clusters among *T. v. cataspila*, reflecting the incomplete differentiation among *T. venusta* subspecies, also revealed by the bifurcating trees (Figs S8, S9).

Some sequences of North American *Trachemys* taxa cluster in the phylogenetic analyses either with the West Indian *Trachemys* species or with *Pseudemys*. It is speculative whether the latter finding reflects ancestral polymorphism or past hybridization. *Pseudemys* is widely sympatric with *Trachemys* in the southeastern USA (compare the maps in TTWG 2021) and it is well known that even very distantly related chelonians are capable of successful hybridization (e.g., *Graptemys* x *Trachemys*; Gooley et al. 2016). It is noteworthy that males of North American and West Indian *Trachemys* share the same sexually dimorphic traits (elongated foreclaws) and the innate courtship behavior consisting of claw vibrations ("titillation") in front of the female's head. In contrast, this courtship behavior is unknown in Central and South American *Trachemys*, in which males do not have greatly

elongated foreclaws. However, in some Central and South American taxa males have prominent elongated and upturned snouts, emphasizing head bobbing movements during courtship (Fritz 1990; Seidel and Fritz 1997).

The presence of the titillation behavior is a plesiomorphic character state since it also occurs in other genera (in particular in *Chrysemys*, *Graptemys*, and *Pseudemys*), while its loss is an autapomorphy of Central and South American *Trachemys* which still sporadically display claw titillation in another behavioral context (aggressive male-male encounters; Fritz 1990, 1991; Seidel and Fritz 1997; Seidel and Ernst 2017).

Compared to our nuclear DNA dataset, phylogenetic analyses of faster evolving mtDNA sequences delivered more information, which albeit reflects only matrilinear evolution confounded by mitochondrial introgression or capture. Except for the above-mentioned unexpected placement of *T. nebulosa*, our mitochondrial trees (Figs 4, 5) contain *Trachemys* as a well-supported monophylum with *T. ornata* and *T. yaquia* together as a deeply divergent mitochondrial lineage. *Trachemys ornata* and *T. yaquia* are reciprocally monophyletic; their genetic divergence resembles those between the three subspecies of *T. grayi* or within *T. venusta*. In contrast to the weak mitochondrial divergence of *T. ornata* and *T. yaquia*, the two taxa appear well separated in our analyses of nuclear DNA (Figs 6, S8, S9), supporting their recognition as distinct species.

In the mitochondrial trees, not all subspecies of *T. venusta* are reciprocally monophyletic, in contrast to the subspecies of *T. dorbigni* and *T. grayi* (Figs 4, 5). The sequences of the subspecies of *T. venusta* are scattered across a polytomy in which also *T. taylori* is embedded. Both the mitochondrial (Figs 4, 5) and the nuclear analyses (Figs S8, S9) fail to identify *T. taylori* as a lineage distinct from *T. venusta*. This conflicts with the current status of *T. taylori* as a distinct species. Using ND4 and R35 sequences, Parham et al. (2015) could not resolve the phylogenetic position of *T. taylori*, and the placement of sequences labeled as *T. taylori* in the nuclear phylogeny of Thomson et al. (2021) is questionable, as misidentified samples appear to have been used (Fritz et al. 2023). Our present data strongly suggest that *T. taylori* does not represent a distinct species and either is a disjunct population of *T. venusta*, perhaps of *T. v. cataspila*, a morphologically similar and geographically close taxon, or a subspecies that recently diverged from *T. venusta*. Notably, based on general morphology, Legler and Vogt (2013: 259) concluded that *T. taylori*, *T. v. cataspila* and *T. v. venusta* are most closely related. Further research is needed to clarify this situation in detail, but in the light of the current data we propose to treat *T. taylori* as a subspecies of *T. venusta*. Using SNP data, Espindola et al. (2022) revealed *T. taylori* as sister to a clade comprised of *T. v. venusta* and *T. v. cataspila*, without discussing taxonomic implications. The branch lengths in their phylogram do not contradict our classification.

Trachemys venusta taylori **nov. comb.** is endemic to the endorheic Cuatro Ciénegas Basin of Coahuila, Mexico, from where two further endemic turtle taxa have been described, *Apalone spinifera atra*, the black spiny softshell turtle, and *Terrapene coahuila*, the Coahuilan box turtle. While *A. s. atra* was originally described as a distinct species (Webb and Legler 1960), it later turned out that it is genetically much less differentiated than expected (McGaugh and Janzen 2008; McGaugh et al. 2008; McGaugh 2012), leading to its recognition as a subspecies of *A. spinifera* (TTWG 2021). Also, the genetic divergence of the morphologically and ecologically divergent aquatic Coahuilan box turtle (cf. Howeth and Brown 2011; Legler and Vogt 2013) is unexpectedly weak; phylogenetically it is nested within the widely distributed and allopatric *Terrapene carolina* (Martin et al. 2021; Thomson et al. 2021). This suggests that all endemic turtle taxa in the Cuatro Ciénegas Basin are phylogenetically very young.

Within the mitochondrial clade of *Trachemys venusta*, some taxa correspond to distinct subclades (Figs 4, 5: *T. v. callirostris*, *T. v. chichiriviche*, *T. v. taylori*), while others do not (*T. v. venusta*, *T. v. cataspila*, *T. v. iversoni*, *T. v. uhrigi*). In contrast to the latter taxa having contiguous parapatric distributions, *T. v. callirostris*, *T. v. chichiriviche*, and *T. v. taylori* are allopatric (compare maps in TTWG 2021 and our Fig. 1). It is remarkable that some of the mitochondrially not clearly distinct taxa appear relatively well-separated in our SplitsTree analysis of nuclear DNA sequences with less than 5% missing data (Fig. 6). However, when the SplitsTree analysis is compared to the remaining phylogenetic analyses of nuclear data, the distinction is no longer so clear (Figs S8, S9). Nevertheless, that some samples appear distinct could indicate an incipient differentiation which is not reflected by mtDNA. This is unexpected considering the much slower pace of nuclear DNA evolution. Possibilities that could have contributed to this confusing pattern are mitochondrial introgression, selective sweeps, or the translocation and subsequent hybridization of turtles.

Several of our samples identified as *T. v. venusta* originate from the region of Acapulco de Juárez, a tourist destination where multiple introductions of *T. venusta* have been inferred (Parham et al. 2015; Fritz et al. 2023). This is also underlined by the photos of three live turtles from Acapulco published in Parham et al. (2015: fig. 3) matching specimens from Tamaulipas (*T. v. cataspila*) in having an irregular carapacial coloration with or without small ocelli and broken postorbital stripes. Our museum specimens from Acapulco (MTD D 39071, 39077, 42598) have in contrast complete well-developed carapacial ocelli and wide continuous postorbital stripes, matching the nominotypical subspecies. This supports that anthropogenic admixture plays a role. However, introductions alone cannot explain our genetic results because we also sequenced museum specimens of *T. v. cataspila* and *T. v. venusta* that have been collected between 1936 and 1955 (Table S1), presumably long before human-mediated long-distance translocations of slider turtles happened. These specimens were genetically also not distinct. Further research, preferably using more informative nuclear genomic markers and more samples, is needed to clarify this intricate situation.

An inspection of the external morphology of our sequenced museum specimens and published known-locality photographs revealed that some individuals display unexpected traits. While most of our specimens of *T. v. uhrigi* (identified according to their collection sites) show the diagnostic characters highlighted in the original description (narrow postorbital stripes, large dark pattern covering most of the plastron; McCord et al. 2010), two have wide postorbital stripes and a narrow dark plastral pattern (MTD D 41609, Honduras; SMF 77494, Nicaragua). This is in line with the observations by McCranie (2018) who studied the morphology of many putative *T. v. uhrigi* from Honduras and concluded "the diagnostic characters given for that nominal form [*T. v. uhrigi*] … conceal the fact that much more variation occurs in Honduran specimens."

It is clear that further research is needed to examine whether the coloration and pattern traits used by McCord et al. (2010) to tell apart subspecies of Central American sliders represent more than population-specific or even individual variation which has been overestimated by cherry-picking morphologically matching turtles. That these coloration and pattern traits are taxonomically unreliable is supported by photos of two live sliders from Dziuché, Quintana Roo, Mexico, and two preserved specimens from Puerto Morelos in the same Mexican state published in Legler and Vogt (2013: figs 40.5 and 40.6). According to the collection sites, these turtles represent *T. v. iversoni*. However, they show narrow to extremely narrow postorbital stripes as described by McCord et al. (2010) for *T. v. uhrigi*. The plastra of the two preserved sliders have a narrow dark figure, whereas McCord et al. (2010) characterize *T. v. iversoni* by a "greatly expanded plastral pattern." Another two live *T. v. iversoni* figured in TTWG (2021: 169) from Cobá, Quintana Roo, and Muná, Yucatán, Mexico, resemble the specimens depicted in Legler and Vogt (2013). Our only studied and sequenced museum specimen of *T. v. iversoni* (SMF 70537) has a nearly uniform yellow plastron. It only yielded mtDNA sequences, and these were not differentiated from those of nine *T. v. venusta* and *T. v. uhrigi*.

Pictures of another two sliders from Alvarado, Veracruz, Mexico, in Legler and Vogt (2013: figs 40.1 and 40.2, according to collection site *T. v. venusta*), show that in the same population individuals with narrow and wide postorbital stripes and very different carapacial ocelli may occur. Also, the lacking or weak genetic differentiation of the subspecies of another slider species, *T. scripta*, and among the species and subspecies of two closely related turtle genera, *Graptemys* and *Pseudemys*, argue for caution (Spinks et al. 2013; Praschag et al. 2017; Vamberger et al. 2020). This situation underlines the concerns voiced in Fritz et al. (2023) that the conspicuous and elaborate color pattern of sliders and their kin resulted in the recognition of taxa that merely reflect population-level variation, a phenomenon of taxonomic inflation also known from other biota with a complex external morphology (e.g., beetles, butterflies, mollusks; Páll-Gergely et al. 2019).

Our analyses of mitochondrial and nuclear DNA sequences do not unambiguously support the distinctness of many subspecies of *T. venusta* (including *T. v. taylori* **comb. nov.**), while the three currently recognized subspecies of *T. grayi* are distinct in mtDNA. Remarkably, both *T. v. uhrigi* and *T. v. iversoni* were ignored in the monograph on the freshwater turtles and tortoises of Mexico by Legler and Vogt (2013) and the respective populations were treated under the nominotypical subspecies *T. v. venusta*.

Although based on subtle differences only, the color patterns of the allopatric South American subspecies of *T. venusta* (*T. v. callirostris*, *T. v. chichiriviche*) and the Mexican subspecies *T. v. cataspila* are easily recognizable, and this is also true for two subspecies of *T. grayi*, *T. g. grayi* and *T. g. emolli* (compare Pritchard and Trebbau 1984; Legler and Vogt 2013; the third subspecies of *T. grayi*, *T. g. panamensis*, is not sufficiently known but may be distinctive as well, see Fritz et al. 2023). However, the putatively diagnostic coloration and pattern traits of the remaining subspecies need to be re-examined and seem to be unreliable to tell apart *T. v. venusta*, *T. v. iversoni* and *T. v. uhrigi*; the same could be true for *T. v. cataspila* and *T. v. taylori* **nov. comb.** Thus, in the face of our genetic results, one option could be to synonymize *T. v. iversoni* and *T. v. uhrigi* under *T. v. venusta* and *T. v. taylori* under *T. v. cataspila*.

Instead, we call for further research using larger sample sizes and preferably genome-wide nuclear markers such as SNPs or low-coverage genome sequencing. In times of large-scale biodiversity loss, the continued use of subspecies names for allopatric and parapatric populations will help prevent inadvertent admixture and erosion of biodiversity when confiscated turtles are released or during conservation measures (ex-situ breeding, population reinforcements) until a better scientific foundation allows for solid evidence-based conservation decisions. Indeed, analyses of SNP data for *T. v. venusta*, *T. v. cataspila* and *T. v. taylori* (Espindola et al. 2022) support that these taxa are distinct.

In a similar vein, more research is also needed to examine whether the two currently recognized subspecies of *T. nebulosa* are distinct. Their recognition is largely based on their allopatric distribution ranges (Legler and Vogt 2013) with the mainland subspecies *T. n. hiltoni* in the Río Fuerte drainage while the nominotypical subspecies occurs on the Baja California Peninsula (Fig. 1). The putatively diagnostic traits (plastral coloration, pygal bone shape; Seidel 2010) are subtle and seem to have never been systematically examined. Our genetic data allow no firm conclusions. Parham et al. (2015) found one individual of *T. n. nebulosa* slightly different from four specimens of *T. n. hiltoni* using combined analyses of the mitochondrial ND4 gene and the nuclear R35 intron. However, even though this matches our results, larger sample sizes are needed both for examining genetic and morphological differentiation before conclusions about the validity of the taxa can be drawn. Legler and Vogt (2013: 299) discussed that *T. n. nebulosa* and *T. n. hiltoni* either represent natural relicts of a formerly more widely distributed taxon around the entire Gulf of California or that the Baja California population (*T. n. nebulosa*) originates from natural or human-mediated dispersal across

the Gulf. However, since the range of *T. yaquia* would separate a continuous range along the Gulf coast, a subsequent range disruption cannot explain the current distribution pattern (compare Fig. 1). An alternative reverse scenario would be *T. nebulosa* originated on the Baja California Peninsula and crossed from there the Gulf to the mainland.

Conclusions

Our present study could not clarify the entangled systematics of slider turtles. However, it contributed some valuable new insights:
(**i**) During the early diversification of *Trachemys*, *T. nebulosa* has captured an alien mitogenome that acts as a genetic poltergeist causing phylogenetic noise in analyses using mtDNA sequences alone or in combination with nuclear data.
(**ii**) The foreign mitogenome of *T. nebulosa* could originate either from the ancestor of the distantly related diamondback terrapin *Malaclemys terrapin* or its extinct sister taxon.
(**iii**) It remains unclear whether *T. n. nebulosa* and *T. n. hiltoni* represent distinct taxa or whether they originate from human-mediated or natural long-distance dispersal across the Gulf of Mexico. However, it is unlikely that *T. nebulosa* once was distributed all around the Gulf, because this range would have been interrupted by the occurrence of *T. yaquia*. A possibility could be that *T. nebulosa* originated on the Baja California Peninsula and spread from there to the mainland Río Fuerte drainage.
(**iv**) Besides *T. nebulosa*, there are six additional deeply divergent and monophyletic mitochondrial lineages that correspond to (1) *T. venusta*, (2) *T. ornata* + *T. yaquia*, (3) *T. grayi*, (4) *T. dorbigni* + *T. medemi*, (5) *T. gaigeae* + *T. scripta*, and (6) West Indian *Trachemys*. These lineages are also supported by our nuclear markers.
(**v**) For *T. gaigeae*, another much younger mitochondrial capture event is likely because its mitogenome is sister to *T. scripta*, although *T. gaigeae* is highly divergent in nuclear markers and resembles Mexican, Central and South American *Trachemys* species in morphology, sexual dimorphism and courtship behavior.
(**vi**) *Trachemys ornata* and *T. yaquia* are distinct taxa with weak mitochondrial divergence, resembling intraspecific mitochondrial divergences in other *Trachemys* species. However, they differ in our nuclear DNA analyses, supporting their species status.
(**vii**) *Trachemys taylori* is neither distinct in our mitochondrial nor nuclear DNA markers and could be a recently isolated population of *T. venusta*. We conclude that *T. taylori* is conspecific with *T. venusta* and identify it as the subspecies *Trachemys venusta taylori* **nov. comb.** This classification is in line with recently published SNP data (Espindola et al. 2022) that reveal *T. v. taylori* as distinct and place it sister to *T. v. venusta* and *T. v. cataspila*.
(**viii**) The number of currently recognized subspecies in Mexican and Central American *T. venusta* is most likely overestimated. Coloration and pattern traits used for diagnosing subspecies are unreliable and could represent population-specific or even individual variation. Further research using more informative nuclear genomic markers and a re-examination of external morphology are needed to lay a solid taxonomic foundation for any conservation strategy.

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Supplementary Material 1

Table S1

Authors: Fritz U, Herrmann H-W, Rosen PC, Auer M, Vargas-Ramírez M, Kehlmaier C (2024)
Data type: .xlsx
Explanation notes: Samples and DNA sequences used in the present study.
Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.
Link: https://doi.org/10.3897/vz.74.e125958.suppl1

Supplementary Material 2

Tables S2–S10

Authors: Fritz U, Herrmann H-W, Rosen PC, Auer M, Vargas-Ramírez M, Kehlmaier C (2024)
Data type: .pdf
Explanation notes: **Table S2.** Applied changes to DNA extraction protocol of Patzold et al. (2020). — **Table S3.** PCR primer pairs for amplicon sequencing and bait-library preparation and their PCR conditions. — **Table S4.** Results of contamination screening using FastQScreen (Wingett et al. 2018) for the obtained quality-filtered reads of sample SMF 22291 (*Trachemys venusta cataspila*) to assess endogenous DNA content in relation to potential contamination sources. — **Table S5.** Mapping details for the samples processed with Next Generation Sequencing. — **Table S6**. The best evolutionary models and partitioning schemes for the mitochondrial dataset as determined by PartitionFinder2 applying the greedy search scheme and the Bayesian Information Criterion. — **Table S7.** Data blocks of the mitochondrial DNA alignment used for phylogenetic analyses. — **Table S8.** The best evolutionary models and partitioning schemes for the nuclear dataset as determined by PartitionFinder2 applying the greedy search scheme and the Bayesian Information Criterion. — **Table S9.** Data blocks of the nuclear DNA alignment used for phylogenetic analyses. — **Table S10.** Comparison of the molecular clock estimates (in million years ago) obtained with the concatenated nuclear and mitochondrial DNA dataset (Fritz et al. 2012) and the mtDNA dataset only (this study).
Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.
Link: https://doi.org/10.3897/vz.74.e125958.suppl2

Supplementary Material 3

Figures S1–S9

Authors: Fritz U, Herrmann H-W, Rosen PC, Auer M, Vargas-Ramírez M, Kehlmaier C (2024)
Data type: .pdf
Explanation notes: Table S1. D1000-TapeStation plot of the single-stranded sequencing library of sample SMF 22291 (*Trachemys venusta cataspila*) after two rounds of in-solution hybridization capture. — **Figure S2.** Scaled assembly for the mitogenome of sample SMF 22291 (*Trachemys venusta cataspila*) as seen in Tablet. — **Figure S3.** Lengths of 549,515 mapped mitochondrial reads of sample SMF 22291 (*Trachemys venusta cataspila*) ranging from 35 bp to 143 bp, with an average read length of 65 bp. — **Figure S4.** Misincorporation plot generated with mapDamage 2.0 (Jónsson et al. 2013) for reads of specimen SMF 22291 (*Trachemys venusta cataspila*) mapped to a published mitogenome of *Trachemys scripta elegans* (KM216748). — **Figure S5.** Scaled assemblies for the nuclear loci of sample SMF 22291 (*Trachemys venusta cataspila*) as seen in Tablet. — **Figure S6.** Lengths of 3158 mapped nuclear reads of sample SMF 22291 (*Trachemys venusta cataspila*) ranging from 35 bp to 143 bp, with an average read length of 60 bp. — **Figure S7.** Divergence time estimates for *Trachemys* and related taxa using a concatenated mtDNA alignment (3221 bp; 12S, ND4L, ND4, cyt *b* plus adjacent tRNA-Thr) and the same settings and fossil calibration points as in Fritz et al. (2012). — **Figure S8.** Nuclear phylogeny of *Trachemys* species and related taxa as inferred by RAxML 8.0.0 rooted with *Deirochelys reticularia* based on phased sequences of five nuclear loci (Cmos, ODC, R35, Rag1, Rag2, 3396 bp, 106 specimens). — **Figure S9.** Nuclear phylogeny of *Trachemys* species and related taxa as inferred by MrBayes 3.2.6, rooted with *Deirochelys reticularia* based on phased sequences of five nuclear loci (Cmos, ODC, R35, Rag1, Rag2, 3396 bp, 106 specimens).
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Link: https://doi.org/10.3897/vz.74.e125958.suppl3