Conserv Genet
DOI 10.1007/s10592-011-0225-x

RESEARCH ARTICLE

Patterns of genetic diversity in the critically endangered Central American river turtle: human influence since the Mayan age?

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Received: 31 May 2010 / Accepted: 5 May 2011

Abstract We conducted a phylogeographic analysis of the strictly aquatic and critically endangered Central American river turtle, *Dermatemys mawii*, as part of a conservation management program for the species. We sampled 238 individuals from 15 different localities throughout the species range. Using sequence fragments from the mtDNA Cyt *b* and ND4 genes, we identified 16 different haplotypes. Overall, our results reveal a signal of phylogeographic structure throughout the range, which appears to have been secondarily blurred by extensive gene flow. Notably, this also applies to genetic structuring across three major hydrological basins that pose biogeographic breaks in other aquatic taxa. Divergence times of mtDNA haplotypes in *D. mawii* suggest that the main lineages split in the Pliocene–Pleistocene (3.73–0.227 MA) and demographic tests indicate that the species has undergone drastic demographic size fluctuations since this time period. One ancient haplotype (1D) was found to exhibit sequence divergence of up to 2% from other haplogroups. Divergence of this magnitude is indicative of species level differentiation in other turtle genera. Haplotype 1D was found in only two localities, Sarstun and Salinas, but specimens with other haplotypes were also found in those localities. It is not known whether the individuals with the 1D haplotype interbreed with non-1D individuals. Our results suggest that human activity, such as harvesting and long distance transport of animals, may have influenced the current patterns of genetic diversity. For more than 2000 years, *D. mawii* has been consumed by people from Middle American cultures, and the archeological record contains strong evidence that the Mayans transported animals between villages and far away from their natural distribution range. Therefore, the large-scale pattern of haplotype sharing even across hydrological barriers, the observed low haplotype diversity in some populations and the contemporary absence of a pronounced phylogeographic pattern is likely due to a combination of population expansions, gene flow, extensive human-mediated-movements and recent bottlenecks resulting from over-harvesting.

Keywords Population genetic structure · *Dermatemys mawii* · Dermatemydidae · Mitochondrial DNA · Freshwater turtles

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Introduction

Two thirds of all surviving species of freshwater turtles and tortoises are threatened with extinction due to habitat destruction, over-exploitation and global climate change (Turtle conservation Fund 2002). The Central American river turtle, *Dermatemys mawii*, is the last surviving species of the giant river turtles of the family Dermatemydidae. It is currently the most endangered turtle species in Central

Published online: 17 May 2011

America and was listed as a critically endangered species in 2005 by the IUCN (Vogt et al. 2005). For centuries, this species has been a part of the diet of the Mayans and other indigenous people who lived in its historic distribution range, which includes the Mexican states of Campeche, Chiapas, Quintana Roo, Tabasco, and Veracruz, Belize, and the Atlantic coast of Guatemala (Fig. 1). In the 1970's, populations of river turtles in Tabasco gradually became scarcer which led hunters to collect animals in the more remote areas at the base of the Yucatan peninsula in Chiapas. Although there have been limited population studies of the species, surveys conducted in 2002 (CONABIO 2009) suggested dramatic recent declines in the populations. Using the exact same techniques used to assess the status of the species a decade earlier, this survey yielded only a fifth of the number of specimens. Most local populations have disappeared and the species is now largely restricted to remote areas inaccessible to humans. The recent increase of a commercial market for its meat has pushed it to the brink of extinction (Vogt, unpublished).

Existing turtle farms in Mexico could play an important role in the captive conservation management of this species. Unfortunately, current management practices have not kept accurate records of the geographic origin of the animals brought into the farms, and captive animals have been bred without consideration to the potential detrimental effects of inbreeding and/or outbreeding depression. In order to design conservation management efforts for captive and wild *D. mawii*, it is important to define the different population units that could merit separate management. Evolutionary Significant Units (ESUs) are groups that exhibit distinct adaptive variation, while Management Units (MU) are those populations that are demographically independent but do not exhibit evolutionary distinction (e.g., Moritz 1994; Crandall et al. 2000). MU's represent populations that are important for the long-term persistence of an entire ESU or species. The conservation of multiple populations is critical for ensuring the long-term persistence of species (Hughes et al. 1997; Hobbs and Mooney 1998). Demography, natural history

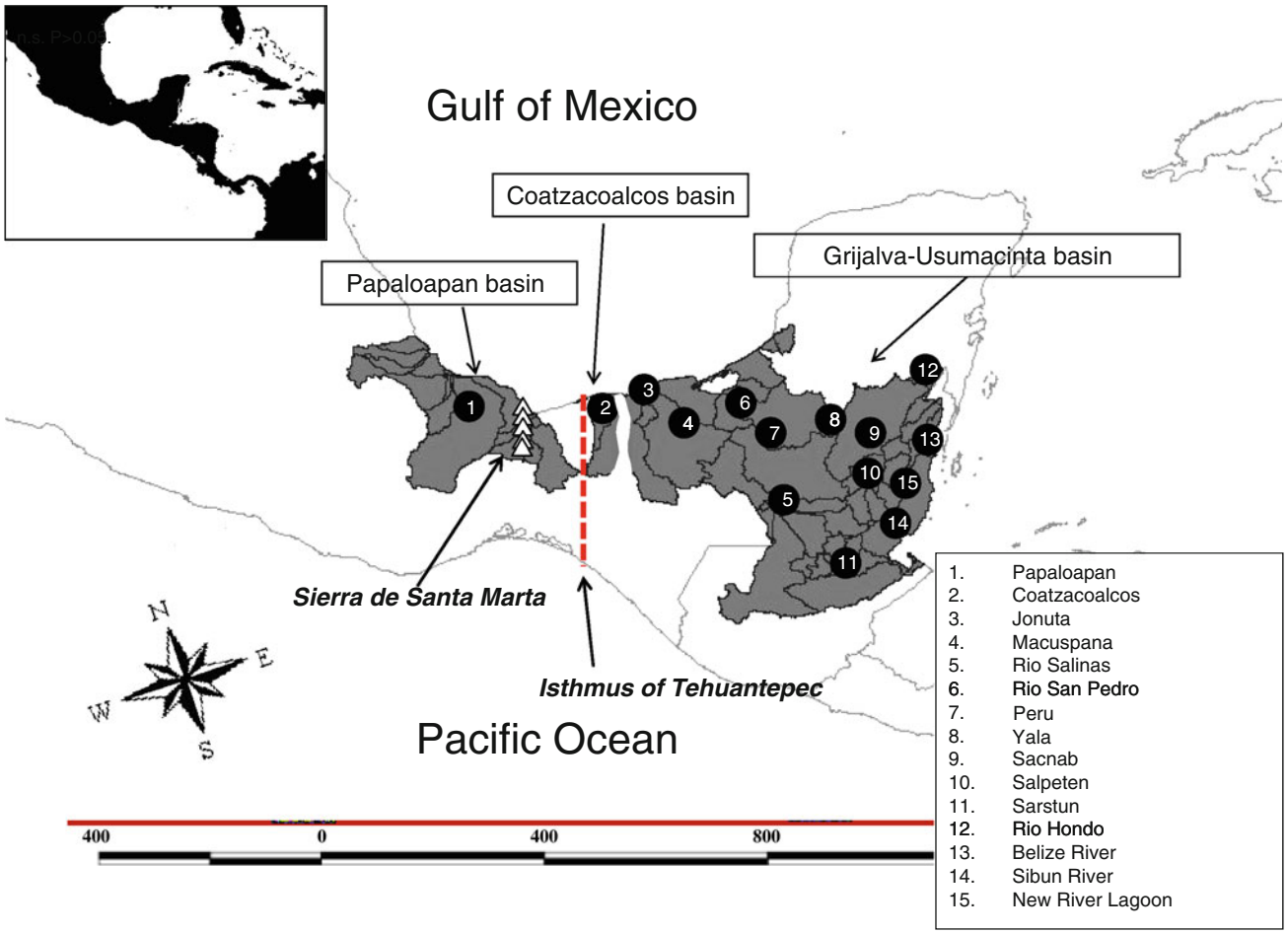

Fig. 1 Geographic distribution of *Dermatemys mawii* (modified from Bulhmann K. 2005). The different main basins are denoted as well as two major barriers, the Isthmus of Tehuantepec and the Sierra de Santa Marta. Sampling localities are: *1*. Papaloapan, *2*. Coatzacoalcos, *3*. Jonuta, *4*. Macuspana, *5*. Salinas, *6*. San Pedro, *7*. Peru, *8*. Yala, *9*. Sacnab, *10*. Salpeten, *11*. Sarstun, *12*. Hondo River, *13*. Belize River, *14*. Sibun, *15*. New River Lagoon. Note that Papaloapan is located more than 100 kms north of the Isthmus of Tehuantepec

and behavioral ecology are important factors to consider because in conjunction with genetic analysis, they provide fundamental information that can help guide conservation strategies and have long-term consequences on management decision making (Keogh 2009).

Few studies have addressed phylogeographic patterns in species overlapping in range with *D. mawii*, but current knowledge tends to predict the presence of phylogeographic structure. Since *D. mawii* is a fully aquatic species, it is limited almost exclusively to freshwater systems (Campbell 1989). Consequently, we would anticipate significant flow of haplotypes along connected systems (within river basins), and far more limited flow between river basins (regions separated by hydrological barriers such as mountain chains or arid land). We collected individuals from three main river basins; the Papaloapan, Coatzacoalcos and the Grijalva-Usumacinta basins. In addition, two major biogeographic barriers known to impede gene flow in other species occur within their distributional range: (a) the Isthmus of Tehuantepec that acts as a biogeographic break for a variety of taxa (Guevara-Chumacero et al. 2010), and the Sierra de Santa Marta, that separates aquatic species of the Papaloapan basin from those in the other basins (Gonzalez Soriano et al. 1997; Rico et al. 2008; Mulcahy et al. 2006). For *D. mawii* we would anticipate genetic structure similar to that found in other vertebrates limited to lowland wetlands.

We assess phylogeographic structure in *D. mawii* and whether significant differentiation exists among different river drainages within its distribution. This will help determine evolutionary and management units, enabling us to make recommendations for the conservation management of this critically endangered species. We hypothesize that because *D. mawii* is fully aquatic, there should be little gene flow between drainages and, potentially, substantial gene flow and little structure within drainages.

Methods

In this study, we characterize the patterns of genetic variability of *D. mawii* using sequences from the mitochondrial DNA (mtDNA) Cytochrome *b* (Cyt *b*) and nicotinamide adenine dinucleotide dehydrogenase subunit 4 (ND4) genes from individuals captured in 15 localities in Mexico, Guatemala and Belize, covering approximately 80% of the geographic distribution of the species (Fig. 1). These gene regions are commonly used for phylogeographic studies among closely related species of turtles (Bowen et al. 1993; Lenk et al. 1999; Farias et al. 2001; Engstrom et al. 2007; Starkey et al. 2003; Stuart and Parham 2004; Spinks and Shafer 2005). Thus, mtDNA can provide a high degree of resolution in addressing regional-scale questions such as regional patterns of genetic variability (Bowen et al. 1992; Allard et al. 1994; FitzSimmons et al. 1997; Kaska 2000; Lopez-Castro and Rocha-Olivares 2005), taking into account that mtDNA is a single marker and may not be neutrally evolving. In particular, the ND4 gene in turtles has been found to have levels of variability comparable to those found in the control region which is non-coding and is generally the most variable region of the mitochondrial genome (Spinks and Shaffer 2005).

Sampling

We collected 238 samples by cutting small 3 mm^2 pieces of inter-digital tissue with clean scissors. Before cutting, the area was disinfected with alcohol gel at 62%, and, after sampling, we covered the wound with antiseptic "new skin" (8-hydroxyquinoline at 1%) in order to avoid infections. Between each sample, scissors were thoroughly cleaned with a 10% bleach solution to avoid cross individual contamination. Pictures were taken of all individuals for photo-identification purposes. All animals were released at the original places where they were collected. The sampling took place between 2004 and 2009 and covered almost the whole range of geographic distribution of this species (Fig. 1). Samples were collected from the following river drainages: Papaloapan ($n = 19$), Coatzacoalcos ($n = 8$), Grijalva-Usumacinta (Jonuta ($n = 25$), Macuspana ($n = 10$), Salinas ($n = 23$) and San Pedro ($n = 20$) rivers, Yala ($n = 19$), Peru ($n = 27$), Sacnab ($n = 27$), and Salpeten ($n = 24$) lagoons, Sarstun ($n = 6$), Hondo ($n = 12$), Belize ($n = 9$), and Sibun ($n = 7$) rivers and New River lagoon ($n = 2$)).

DNA purification, amplification and sequencing

Immediately after collection, tissues were preserved in 70% ethanol and stored in a −80°C freezer. DNA was extracted from tissues using the DNeasy extraction kit (QIAGEN). Then, polymerase chain reactions (PCRs) were performed using mtDNA Cyt *b* primers L14724 (TGTAA AACGACGGCCAGTTGTGTAGTATGGGTGGAATGG) (Irwin et al. 1991) and H15149 (ACTGCAGCCCCTCAG AATGATATT TGTCCTCA) (Kocher et al. 1989). Initially, we attempted to amplify an 890 bp fragment of the ND4 gene using primers that were designed to successfully amplify other species of turtles (LND4 and HLeu; Stuart and Parham 2004). However, these primer would not consistently amplify samples of *D. mawii*, therefore, we redesigned two species specific primers GPND4-L (CCA AAAACACTCTACTACCCATTCA) and GPND4-H (TG AACAGTGAGAAATACCTCAAAT), using "Primer3" (Rozen and Skaletsky 1999). These primers amplified a shorter fragment and we obtained 575 bp sequences for all

sampled individuals. We used AmpliTaq Gold® DNA polymerase (Roche Molecular Systems, Inc.) for amplifications under the following conditions: Initial amplification at 94°C for 7 min, followed by 45 cycles of denaturing at 92°C for 1 min, annealing at 50°C for Cyt *b* and 60°C for GPND4 for 1 min, extension at 72°C for 1 min, one cycle of extension at 72°C for 7 min and 10°C on hold. Sequencing reactions of the PCR products were conducted using the same primers and the Big Dye® Terminator v3.1 cycle sequencing kit (Applied Biosystems). These products were sequenced directly in a 3130xl genetic analyzer (Applied Biosystems). The resulting sequences were analyzed using Sequencher version 4.1 (Gene Codes). Then these sequences were cleaned by direct alignment, inspected and corrected by eye. We also included two Genbank sequences of *D. mawii* for Cyt *b* and ND4 from Sarstun River in Guatemala (Accession numbers AY678313.1 and AY673524.1).

Finally, we obtained tissue from a common musk turtle (*Sternotherus odoratus*) from the herpetological collection of The University of Texas at Arlington. We selected this species as an appropriate outgroup because it has been previously determined to be one of the most closely related species to *D. mawii* and it belongs to the same superfamily Kinosternoidea (Hutchison and Bramble 1981; Bickham 1981; Bickham and Carr 1983; Rhodin et al. 2009; Fujita et al. 2004).

Phylogenetic analyses

We analyzed 238 sequences for polymorphism for each gene region (Cyt *b* and ND4) of *D. mawii* using DNASP 4.50. 3 (Rozas et al. 2003). Pairwise Kimura 2-parameter distances (Kimura 1980) were estimated and used to construct a neighbor-joining (NJ) tree (Saitou and Nei 1987) using PAUP *4.0 (Swofford 2002). In addition, maximum parsimony (MP) and maximum likelihood (ML) trees were also estimated using PAUP *4.0 (Swofford 2002). One thousand replicates of a heuristic search were performed with an initial random stepwise addition of sequences and tree-bisection-reconnection branch swapping. Branch support was estimated from 10,000 replicates of bootstrap search. Additionally, we performed a Bayesian analysis using MrBayes 3.2.1 (Ronquist and Huelsenbeck 2003). The settings were two simultaneous runs of Markov chain Monte Carlo (MCMC) for ten million generations, sampling every 1,000 generations, for four chains, a heating parameter value of 0.30 and burn-in of 0.5%. For this test, and the maximum likelihood analysis we inferred an evolutionary model using MrModeltest 2.3 (Posada and Crandall, 1998; Nylander 2004). The values for this model were: maximum likelihood $Ln = 2285.780$, $K = 5$, $AIC = 4581.560$. The base estimate frequencies were $A = 0.291$, $C = 0.179$, $G = 0.2092$, and $T = 0.321$. The substitution value = Ti/tv radio = 15.292, the proportion of invariable sites estimate value = 0, and the Gamma = 0.088. In addition, we generated a statistical parsimony haplotype network using TCS 1.21 (Clement et al. 2000). TCS calculates the number of mutational steps among all pairs of haplotypes and then joins the most similar haplotypes together into a network where their combined probability is greater than 95% (Templeton et al. 1992).

Variability within and genetic structure among populations

In order to estimate the genetic variability within populations, we calculated the haplotype diversity and the nucleotide diversity of each population using DNASP 4.50. We performed a series of statistical analyses of the various populations in order to determine if signals of genetic structure are present among populations. First, we tested our hypothesis of isolation of individuals based upon the separation of the river basins from where they were collected. Then, we estimated the significance of geographical divisions among local and regional population groupings using an Analysis of Molecular Variance (AMOVA) included in ARLEQUIN 3.11 (Excoffier et al. 1992). We performed three tests in order to estimate the levels of gene flow between groupings defined by the genetic structure analysis. First, we performed Fisher's exact test of population differentiation to identify which pairwise groupings were significantly differentiated (Raymond and Rousset 1995; Goudet et al. 1996; included in ARLEQUIN 3.11). Second, we evaluated the number of migrant females per population included in DNASP 4.5 03 based on Hudson, et al. (1992). Finally we obtained indirect estimates of gene flow using Φst values. In diploid animals, Φst values represent the allelic frequency variations between populations and, therefore, the genetic differentiation between these populations can be estimated from the formula Fst = 1/(4Nm + 1). F_{ST} values have been used to obtain estimates of gene flow in previous studies (e.g. Stanley et al. 1996; Maldonado et al. 2001), however, they rely on numerous assumptions and have to be taken with caution (see Whitlock and McCauley 1999). We also assessed differentiation by distance by plotting average number of nucleotide differences (log values) versus geographic distance values (also log values). The significance of this correlation was assessed by generating a probability distribution with 1,000 permutations using the program IBDWS (Isolation by Distance Web Service) Version 3.16 (Jensen et al. 2005).

Divergence times between lineages

Sequences of mtDNA can be used to estimate divergence time between populations when corrected for ancient polymorphism (Nei 1987; Maldonado et al. 2001). These estimates of divergence time are only approximations; recent gene flow can cause underestimation of the divergence times among populations, especially if the time of divergence was far in the past (Arbogast et al. 2002). We thus estimated the percentage of nucleotide substitutions between the main mtDNA lineages. Since mtDNA mutation rates in turtles are highly variable, they can exhibit the conventional 2% per million years (1×18^{-8} substitutions/year/nucleotide position) reported for several species of mammals, birds, fish, and even *Drosophila sp*. (Avise 1992). However, much slower mutation rates of 0.36–0.4% per million years have been reported in turtles, such as in the case of the map turtle of the genus Graptemys (Lamb et al. 1994), and 0.4% for Testudines (Bowen et al. 1992). We therefore employed two estimates of mutation rates, 2 and 0.4% per million years, using the percentage of nucleotide substitutions between populations.

Tests of neutrality and demography

Neutrality tests were performed in ARLEQUIN 3.11 using Tajima's D (1996). This statistic compares the total number of segregating sites within a sample to the average number of pairwise nucleotide differences. If these two values are equal, the genetic drift within the sample is deemed random or neutral. If the two values diverge by more than can be attributed to chance, changes are deemed to be non-random (Tajima 1996). We also performed a Fu's Fs (Fu 1997) analysis for each population to explore for signals of demographic fluctuations.

Results

We sequenced a 420 bp fragment of the Cyt *b* which defined 7 haplotypes. These haplotypes were designated numbers 1–7. Sequencing of a 575 bp fragment for the ND4 region yielded 9 haplotypes. These haplotypes were designated letters A–J. (Genbank Accession numbers: Cyt*b*. haplotypes 1-7: HQ709246, to HQ709252, respectively), and haplotypes: ND4 A–J: HQ709253–HQ709263, respectively). We converted these haplotypes into amino acid sequences to check for stop codons to confirm that we had amplified a mtDNA functional gene and not a nuclear insert or pseudo-gene. Because the Cyt *b* and ND4 genes evolve at roughly equivalent rates (Spinks and Shaffer 2005), we concatenated sequences from both regions to produce a 997 bp fragment for 238 individuals, resulting in 16 different combined haplotypes (Table 1; Fig. 2). Within the combined 997 bp fragment we found a total of 35 polymorphic sites.

Table 1 Mitochondrial DNA haplotypes (in rows) and their frequency in each locality (columns)

	Pap	Coa	Mac	Jon	Sal	SP	Yal	Per	Salp	Sac	Sar	Hon	Bel	Sib	NR	Total
1D					2						3					5
2A	14	8	2	2	9						1					36
2I	1															1
3A			2	1	2											5
4E	1															1
5A	1		2	18	6	10	4	24	6	27	1	2	9	7	2	119
5B			1	1	2											4
5C			1		1											2
5E	1															1
5F			1	1					1							3
5G			1					1			1					3
5H									6							6
5J						10	15	2	11			10				48
6A				2												2
7A					1											1
7E	1															1
	19	8	10	25	23	20	19	27	24	27	6	12	9	7	2	238

Pap Papaloapan, *Coa* Coatzacoalcos, *Mac* Macuspana, *Jon* Jonuta, *Sal* Salinas, *SP* San Pedro, *Yal* Yala, *Per* Peru, *Salp* Salpeten, *Sac* Sacnab, *Sar* Sarstun, *Hon* Hondo River, *Bel* Belize River, *Sib* Sibun, *NR* New river lagoon

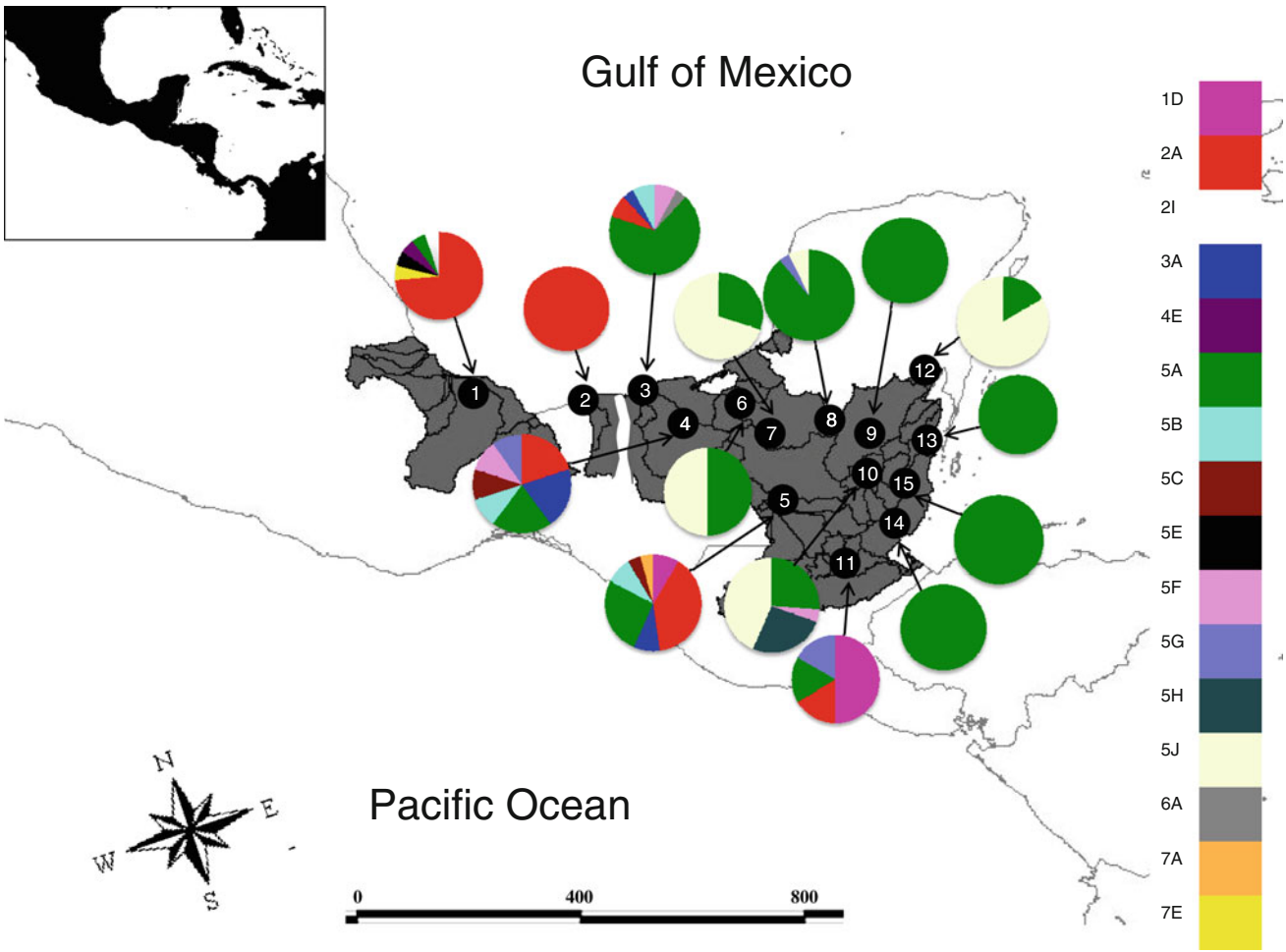

Fig. 2 Haplotype distribution on the geographic range for *Dermatemys mawii*. The *pie graphics* represent the frequencies of haplotypes in each locality

Phylogenetic analysis

We conducted a phylogenetic analysis but it resulted in an unresolved phylogeny. The neighbor-joining, maximum likelihood and maximum parsimony methods of tree reconstruction resulted in a polytomy with a few nodes that had low bootstrap support (Figure not shown). Therefore, we constructed a statistical parsimony haplotype network (Fig. 3) in order to better depict the relationship of haplotypes. The resulting network shows two long branches with haplotypes that are highly differentiated from the haplogroup around the centrally positioned haplotype 5A. On one of the longest branches, there is a highly divergent haplotype (1D) which differs from haplotype 5A by a total of 22 substitutions (2.47% sequence divergence) and was found in the south-east part of the species range, in Salinas and Sarstun. On the other long branch, nine substitutions separate the most divergent of three haplotypes (4E,7E, 5E), which were found only in Papaloapan, in the westernmost part of the range of *D. mawii*. Haplotype "5A" was the most common haplotype, and was present in 119 out of 238 individuals (Table 1; Figs. 2, 3). Notably, this haplotype was found in almost all localities except in the Coatzacoalcos population, thus spanning the entire distribution range of *D. mawii*. The Coatzacoalcos population did not appear to be an outlier, since it showed a haplotype (2A; the third most common haplotype, found in 36 individuals) that was also found throughout the sampled range. The second most common haplotype, 5 J (also from the centrally placed haplogroup), was present in 48 individuals and concentrated to populations in the central part of the range (San Pedro, Yala, Peru, Salpeten and Hondo river). The remaining 9 haplotypes (2I, 3A, 5B, 5C, 7A, 5F, 5G, 5H, and 6A) were rare, found in only 1–6 individuals each. Those haplotypes, in conjunction with 2A, 5A and 5J show a star-shaped

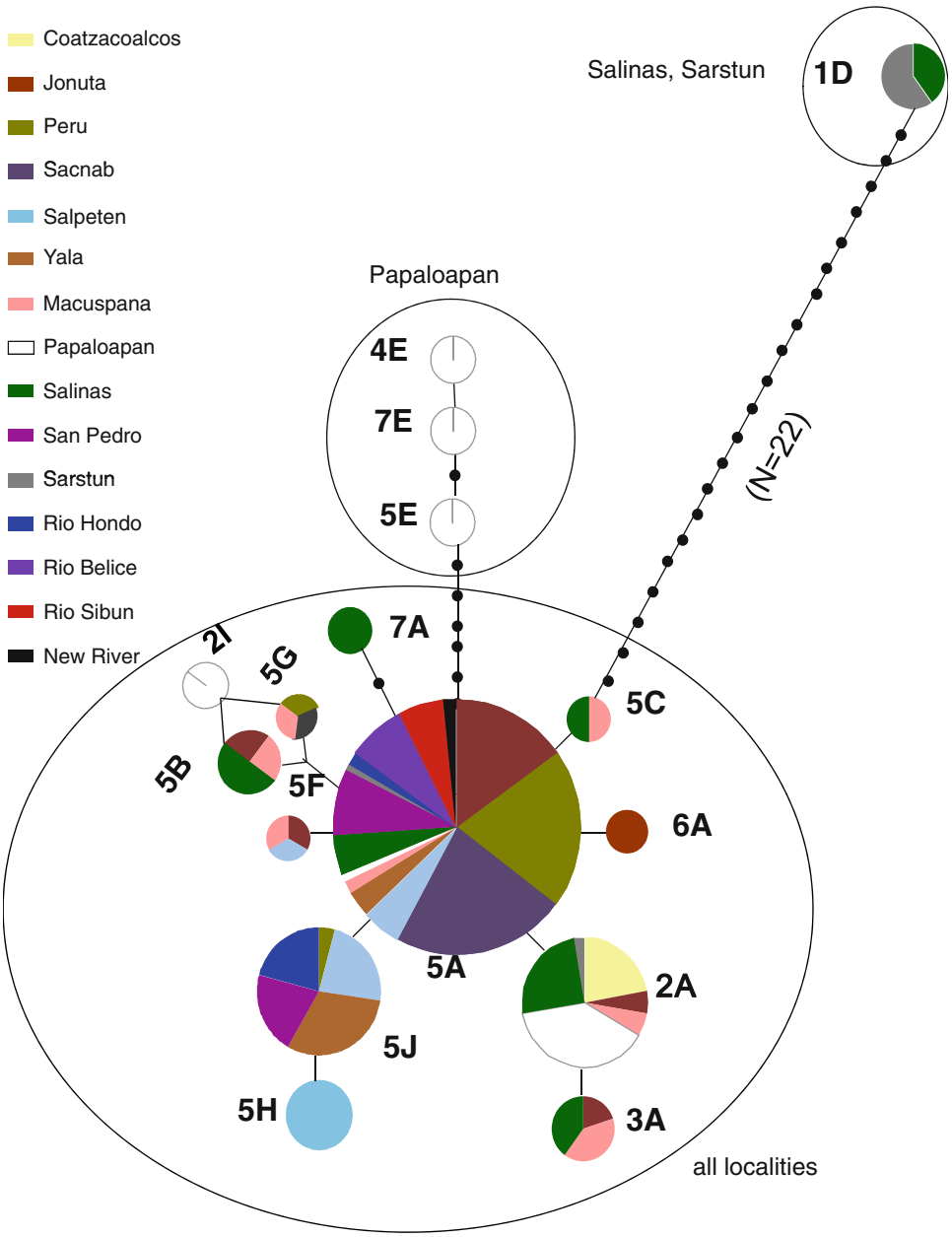

Fig. 3 Statistical parsimony network obtained from TCS (Clement et al. 2000), based on a 95% connection limit. The figure shows three different haplogroups or genetic lineages (Central, 1D, and unique from Papaloapan (PAP), and the localities where these haplogroups occur)

Table 2 Mitochondrial DNA diversity within *D. mawii* lineages

Lineage	n	N_H	*HD*	Π	Fu's F_S
Central	230	12	0.651	0.001	−2.98*
PAP	3	3	1	0.002	0.98
1D	5	1	0	0	0

n is the number of sequenced individuals, N_H the number of distinct haplotypes, *HD* haplotype diversity, π nucleotide diversity (both ± standard errors) and Fu's F_S

* $P < 0.05$

arrangement with only one to three substitutions from the central haplotype 5A (Fig. 3) suggesting a recent divergence and rapidly expanding populations.

Variability within lineages or haplogroups

The distribution of haplotypes found in the three main lineages was as follows: The central lineage was integrated by most haplotypes with the exception of 1D, and 4E, 5E, and 7E and for the rest of this paper will be called "Central", the one integrated by only haplotype 1D will be called "1D", and the one integrated by the haplotypes unique to Papaloapan, will be called "PAP". The Central haplogroup showed the largest number of haplotypes (12) in 230 individuals, while the PAP showed just 3 haplotypes with 3 individuals, one for each different one, and 5 individuals had the 1D haplotype (Table 2). Haplotype diversity within each of the three lineages ranged from 0 in 1D

to 1 in PAP, and the nucleotide diversity ranged from 0.000 in 1D to 0.002 in PAP (Table 2).

Gene flow and genetic structure between populations

When we tested the hypothesis that *D. mawii* populations exhibit genetic structure between the three different hydrological basins using AMOVA, the results were not significant ($P > 0.05$) indicating the absence of significant barriers to gene flow over the recent evolutionary past. We subsequently analyzed genetic structure among *D. mawii* populations using SAMOVA 1.0 (Spatial Analysis of Molecular Variance) (Dupanloup et al. 2002). This approach does not require a priori knowledge regarding population groupings. The results of this test indicate that the maximum Φ_{CT} value belongs to the arrangement of two different groups, one including all localities, and another one including only Sarstun as a different population. Furthermore, the high levels of gene flow detected between localities in different river basins separated by long distances and biogeographic barriers (i.e., between Papaloapan and Salinas) or very low levels of gene flow between localities close together (i.e., between Hondo river and Belize river), support the results of the analysis of isolation by distance (Isolation by Distance Web Service, Version 3.16; Jensen et al. 2005) that indicated there was no significant correlation between genetic and geographic distance with $Z = -592.725$, $r = 0.338$ and $P = 0.961$.

Divergence time inferred from sequence data

The average % sequence divergence values between lineages of *D. mawii* ranged from 0.009% (between Central and PAP) to 0.03% (between 1D and PAP). These lineages were estimated to have diverged between 0.227 MYA (assuming a mutation rate of 2% per million years) and 1.125 MA (assuming a mutation rate of 0.4%). The oldest estimated time of divergence was between 1D and PAP. These lineages diverged between 0.752 MA (2% mutation rate) and 3.73 MA (0.4% mutation rate) (Table 3). All of these values fall within the Pliocene–Pleistocene, between 1.125 and 3.730 million of years.

Table 3 Values of divergence time estimated with average number of substitutions per site between populations and two different mutation rates known for turtle species (a) upper right 2%, and (b) 0.4% lower left

0.4%	2%		
	Central	1D	PAP
Central	–	0.577	0.227
1D	2.864	–	0.752
PAP	1.125	3.730	–

The values in the upper right indicate that the divergence between these populations occurred within the Pleistocene between 0.227 and 0.752 million of years at 2% of mutation rate, while at 0.4% the divergence times between the PAP and 1D dates from 3.73 millions of years ago, and 1D and the central haplogroups date 2.864 millions of years both during the Pliocene, and the PAP and central diverged 1.125 million of years ago during the Pleistocene

Neutrality test and demographic analyses

Tajima's D values for the "Central" haplogroup were not significant ($D = -0.83078$, $P = 0.22$ $P > 0.05$). In addition, Fu's (1997) Fs test statistic indicated significant population expansion only for the Central haplogroup (Table 2), the other two lineages were neither significant for Tajima's D nor for Fu's F_S test.

Discussion

This is the first study of freshwater turtle phylogeography and population genetics for the Mesoamerican and Central American regions. It is also the first genetic study of *D. mawii*, the only surviving species of the monotypic family Dermatemydidae. There is very little known about the biology and ecology of this species and it is critically endangered, mainly due to human consumption. In this study, we implemented different genetic analyses in order to assess levels of phylogeographic structure in *D. mawii* and whether significant differentiation exists among different river drainages along the distribution of this poorly known species.

Phylogenetic analysis

Our phylogenetic analyses identified three divergent phylogenetic lineages in the haplotype network. One, constituted by haplotype 1D, it is highly divergent from the others with a genetic divergence of up to 2%. Divergence of this magnitude is indicative of species level differentiation in other turtle genera such as *Graptemys* (Lamb et al. 1994). Animals with haplotype 1D were restricted to Sarstun and Salinas localities, along with specimens having other haplotypes that were also found in other localities. Two different genetic lineages could diverge due to the existence of barriers to gene flow, but if these barriers later disappear, secondary contact could mix the individuals again. The presence of this ancient lineage (1D) mixed with haplotypes from other lineages could be explained by the breakdown of geographic barriers to gene flow as a result of natural or human-mediated causes. The pattern we see could thus be the result of secondary contact and subsequent interbreeding. Alternatively, those could be two genetically isolated lineages that do not currently interbreed. As mtDNA reflects only maternal lineages,

we cannot differentiate between those two hypotheses. Therefore, it is important that future studies use hypervariable bi-parental nuclear markers, such as microsatellites, in order to clarify finer-scale patterns of genetic structure and to determine whether individuals with haplotype 1D are interbreeding with individuals carrying the other more widely distributed haplotypes. The individuals with haplotype 1D did not show apparent external morphological differences (P.G.G.-P, *pers. obs.*), but detailed morphological studies on this are warranted based on our results. Our sample from Sarstun contains only six individuals, three of which have haplotype 1D. It is important to do future surveys along the areas where this haplotype is found (Sarstun and Salinas) in order to search for the presence of other haplotypes that may better explain the history of this lineage. A study of patterns of movement using mark–recapture methods would be warranted, but the fact that *D. mawii* is highly harvested for human consumption makes this almost impossible.

A second lineage in the haplotype network includes haplotypes found only in the Papaloapan river (5E, 7E and 4E) referred to as unique to Papaloapan (PAP) haplogroup. This lineage exhibits divergence levels up to 1%, and also co–occurs with individuals carrying haplotypes that are found in other localities. This could represent a lineage that was the result of historical (possibly pre-human; see below) isolation caused by the Isthmus of Tehuantepec, as the Papaloapan locality is the only one north of this geographic barrier (Guevara-Chumacero et al. 2010; Rico et al. 2008), and the Sierra de Santa Marta, which is at the southern part of the Trans-Mexican Neovolcanic Belt, that separates aquatic species from the Papaloapan basin from those in the other basins (Gonzalez Soriano et al. 1997). Similarly, in the genus Bufo there are different genetic lineages at the west and east to the Isthmus of Tehuantepec and Sierra de Santa Marta (Mulcahy et al. 2006). Even species occurring in apparently homogeneous lowland habitats in Central America can exhibit population genetic structure when movement is inhibited. The red-eyed tree frog (*Agalychnis callidryas*) of lower Central America shows pronounced genetic structure not only across the Cordilleran Mountains, but also along the Caribbean and Pacific coastal forests (Robertson and Zamudio 2009). On the other hand, species with lower habitat specificity and thus greater freedom of movement exhibit less structure: the Coahuilan box turtle (*Terrapene coahuila*) is a semi-aquatic species capable of stepping-stone movement among regional wetlands and exhibits high levels of gene flow (Howeth et al. 2008). While *D. mawii* could show similar high levels of gene flow within drainages, we did not anticipate this pattern when comparing populations which are separated by high mountains and unsuitable habitat for a strictly aquatic species. In summary, our finding of haplogroup Central across different basins and biogeographic regions is unexpected given the strictly limnic habitat requirement of *D. mawii*.

The third Central haplogroup includes the remaining haplotypes and exhibits considerable intra-lineage divergence. Animals carrying those haplotypes were found in all localities of our study area (thus including localities with haplotypes from other mtDNA lineages; Salinas, Sarstun and Papaloapan). The star-shaped arrangement of these haplotypes in the network is coupled to signals of a population expansion (according to Fu's F_S). This overall pattern of haplotype arrangement including one with a star shape and two long branches is similar to the one found in Morelet crocodiles (*Crocodylus moreletii)*, in the same range of distribution of *D. mawii* and has also been heavily exploited by humans (Ray et al. 2004) in which one long branch showed more than 20 changes from the central haplotype, and this central clade has a star shape with one to four changes from the central haplotype, but in this case of *C. moreletii*, the long branch also shows another star-shaped clade with one to four changes between them. The difference with *C. moreletii* is that the genetic variation is well-structured and fits the isolation by distance model and *D. mawii* lacks this clear genetic structure. In fact, *C. moreletii* is not restricted to water and is capable of moving on land contrary to the highly aquatic *D. mawii*. This may explain the higher gene flow among regions observed in *C. moreletii*.

We found that the highest haplotype diversity is concentrated in the western part of the geographic distribution of *D. mawii* (Table 1; Fig. 2). Western localities such as Jonuta, Papaloapan, Macuspana and Salinas contain up to 6 or 7 haplotypes, while most localities in the eastern part have only one or two haplotypes. Some haplotypes like 2A and 3A, are common in the western part of the distribution of this species but absent in the eastern part, and the opposite is true of haplotype 5J, which is common in the east but is absent in the west. Such an east–west gradient in our data is surprising; given that climate throughout the range of *D. mawii* is relatively homogeneous. The region is located in the lowlands of the Gulf of Mexico and the Caribbean, characterized by abundant rainfall and a relatively short dry season and high temperatures throughout the year (West 1964). Consistent with our prediction of high gene flow within drainages, we found that all southeastern populations except Salpeten and Sarstun shared most of their haplotypic diversity. In particular, haplotypes 5A and 5J, which are separated by a single substitution, were found in at least 96% of individuals in this region.

Evolutionary history of *D. mawii*

According to Savage (1966, 1982), the genus Dermatemys was already living in Mexico and Central America 55

MYA during the Eocene; however, there are no known fossils of the genus or the family from Mesoamerica (Carrol 1988; Flores-Villela 1993; Reynoso 2005). Our estimates of sequence divergence between lineages range from 0.227 to 0.752 MY if we use the faster 2% sequence divergence rate, and from 1.125 to 3.73 MY if we use the slower rate of 0.4% (Avise et al. 1992). Despite the large difference between these two rates, we can conclude that the main intra-specific divergence within *D. mawii* occurred during the Pleistocene and/or Pliocene (Table 3). The Pliocene–Pleistocene epoch was characterized by extreme cyclical climatic changes, with glacial and inter-glacial periods and transient cooler and drier periods than at present (Duellmann 1966; Leyden 1984). During the Pliocene the climate for Central America was characterized as a dry and cooling period (Stuart 1957, 1966). During that time, *D. mawii* may have occurred in the Mexican province of Veracruz, (Savage 1966), after it migrated from North America, along the lowlands. During the Pleistocene, the sea levels were down to 100 m below current levels, and due to lower rainfall patterns, the water levels of wetlands like the Laguna Salpeten were much lower than today. Water was only present during the rainy season (Hodell et al. 2008). Areas currently located within the same drainages may therefore have lacked a waterway connection during the cold periods of the Pleistocene, leading to genetic differentiation. The fluctuating climatic conditions during the Pliocene–Pleistocene, with alternating periods of humidity and dryness (Lee 1980), could have had a large impact on the population genetic structure. Under these conditions, populations of this highly aquatic turtle that cannot move on land could have become isolated in the small remaining water drainages during dry periods (Campbell 1989). When the climate became more humid and the drainages expanded, flooding would have enabled population growth and facilitated movement between the lower parts of the rivers allowing turtles to disperse longer distances. Gene flow between different river basins could have occurred through brackish, or even through salt water since *D. mawii* has been occasionally observed to enter brackish or saltwater in Laguna de Términos, Campeche, and Bahia de Chetumal, Quintana Roo (P.G.G.-P., *pers. obs.*, Feb, 2009). A similar dispersal mechanism has been suggested for turtles of the genus *Graptemys*, which are also highly aquatic and whose adult females nest only a few feet from the water (Lamb et al. 1994).

Climatic and geologic conditions in the Grijalva-Usumacinta basin, which covers approximately half of the species range of distribution, were very similar during the Pleistocene to what they are today (Leyden 1984), although they were drier and cooler than at present. In some cases droughts were severe, and some of the lakes in the region (i.e. Salpeten) were dry for a large portion of the year. The dry climatic conditions could have caused severe population bottlenecks causing extinctions, which in turn would result in a drastic reduction in the number of haplotypes with a distribution such as the one we found in Yucatan and Salpeten (Fig. 2). The Grijalva-Usumacinta basin is the only part of the species range with information regarding conditions during the Pleistocene, however, we anticipate that the other basins underwent similar conditions.

Lack of population structure and the potential influence of ancient and modern humans

Although, our sampling covers three river basins which are separated by unsuitable habitat for the species (mountain chains with passes above more than 1,000 m of elevation, like at Sierra de Santa Marta), we found only weak geographic structuring and extensive haplotype sharing among most *D. mawii* populations. Our results revealed a surprising lack of phylogeographic pattern between localities within and between basins. Our results were also characterized by unexpectedly high levels of gene flow between localities in different basins separated by great distances, and low levels of gene flow between closely spaced localities with a lack of statistical support for any pattern of isolation by distance. One hypothetical explanation for this mixed pattern could be that multiple colonisations occurred because, historically, the river basins that they inhabited were much more interconnected than at present or, less likely, these strictly aquatic turtles would have travelled great geographic distances across substantial terrestrial barriers, including mountains, to reach their current localities. Another hypothesis would involve humans transporting turtles long distances for consumption, trade or ritual purposes. Individuals with common haplotypes would be more likely to be captured and dispersed. It remains unclear, however, why the 1D and Papaloapan lineages were not also transported to occupy a larger range today. This may relate to the origin of the trade/transport in a region where the Central mtDNA lineage was prevalent historically, or recent demographic events have masked ancient human-mediated transport of the remaining lineages. It is well-documented that *D. mawii* has been consumed by humans for several centuries and even millennia, and it is possible that these turtles were part of the diet of the Olmec culture more than 3,000 years ago (Soustelle 2003). Turtle species all over the world are appreciated for their value as a source of animal protein; they are relatively easy to feed and raise in captivity as they can be kept alive in small ponds and in remote areas without refrigeration (Jenzen and Das 2008). Indeed, aquatic turtles have been an important source of animal protein for inhabitants of the lowlands of northern Central America since even

before the Spanish could document these practices in the area (Ximenez 1967). The Mayans transported animals to different places either for ceremonial purposes or as food items (Lee 1996). Not surprisingly, *D. mawii* was a very important source of animal protein for the ancient Mayans of the Peten (Preclassic period 800–400 B.C.). There are several references to the presence of remains of these turtles (bones, shells) in the Mayan temples of Uaxactun (Stuart 1958), Tikal, Petexbatun, Las Pacayas (O'Day et al. 2004) and San Jose, Peten, (Emery 2001; Castellanos-Cabrera 2007). Remains of these turtles have been found in Copan in Honduras (Emery 2005), and in Veracruz (Wing, 1976 in Iverson and Mittermeier 1980). Turtle remains were part of burial offerings for people of high status found within the range of the current distribution of this species (Lee 1996). We have also found a reference to a specimen of *D. mawii* in one burial site at the Teotihuacan archeological zone in the state de Mexico, more than 300 km from known distributional range of the specie's (Elson and Mowbray 2005). In addition, a sculpture of this species housed at the Anthropology museum at Mexico City, was discovered in the Basin of Mexico, more than 350 km from the closest locality from its known distribution range.

These practices continue today. In Guatemala, *D. mawii* individuals are kept in medium size ponds called "Aguadas" where these turtle can be easily captured when they are needed (Campbell 1989). Similarly, in the State of Tabasco, Mexico, turtles captured intentionally or as by-catch are kept in rustic ponds and raised until they are either consumed by the fishermen on special occasions or sold for high prices. One kg of *D. mawii* meat can fetch prices of $100 USD (Vogt *pers. comm*).

Documentation exists of accidental releases or escapes (during floods) into the available local water drainages in recent times; some *D. mawii* individuals escaped from the turtle farm in Tucta, Tabasco, Mexico during 2007 floods in the State of Tabasco (Semarnat Tabasco, *pers. comm.*). It is not unlikely that this could have occurred in Mayan times as well. The pattern observed in *D. mawii* is similar to that reported for terrapins (*Malaclemys terrapin)*. This species inhabits coastal tidal marshes throughout the Eastern United States and has been found to lack population genetic structure despite the fact that the terrapin is known to be highly philopatric. This pattern was attributed to extensive translocations of terrapins during the early 20th century to replenish diminished populations and to provide turtle farms with stocks for their high demand in the pet trade (Hauswaldt and Glenn 2005). Artificial movement of *D. mawii*, carried out for hundreds or thousands of years, in combination with bottlenecks caused by overhunting, could explain the odd pattern of haplotype distribution that we observe in this species.

Conservation implications

Populations of *D. mawii* are on the brink of extinction across their entire distribution (Vogt et al. 2005). Although habitats of the species remain in good condition across a portion of its historical range, the remaining populations have undergone recent severe demographic declines mostly due to human poaching (Vogt unpublished; CONABIO-DGVS-CONANP 2006). Therefore, all remaining *D. mawii* populations require special protection. Captive management programs in situ and *ex situ* that meet high standards of record keeping and genetic management should be promoted, in order to retain viable populations for future reintroductions into protected habitat within its historic distribution range (Syed et al. 2007).

Our results showed that there are three defined *D. mawii* haplogroups (Central, 1D and PAP). These three lineages could be considered one management unit. It is important to maintain viable captive populations of animals from this population and to implement effective genetic management of these organisms. Ongoing captive efforts such as turtle farms like the one in "La Florida" and captive turtle centers with conservation purposes like the one in "La Popotera", both in Veracruz, need to be supported. Finally, it is also crucial to protect the wild *D. mawii* populations from this basin through environmental education efforts, and through support for the local authorities (Aguirre 2007).

We also recommend that populations in the Sarstun and Salinas regions should also be managed as an ESU to protect haplotype 1D. Groups having haplotype 1D (Salinas and Sarstun) showed high levels of genetic divergence (2%) with ancient lineages. We further recommend that continued research on these populations be conducted. Such additional genetic analysis should include nuclear loci, in order to assess if the patterns found in this study also hold for biparentally inherited markers. Also, a detailed morphometric analysis looking at patterns of morphological variation throughout the species range should be conducted, since it will be important to determine if the animals carrying the divergent 1D haplotype represent a reproductively isolated lineage. Breeding experiments need to be undertaken to ascertain the reproductive viability of the offspring. Because a number of these animals are located in captivity at the Philadelphia Zoo and the turtle farm in Tucta, Tabasco, these analyses are feasible in the near future.

How has gene flow resulting from turtles that were moved from different populations affected the patterns of genetic variability of the species? As outlined above, the economical importance of *D. mawii* has led to long-range transport of animals over centuries or perhaps even millennia by the different cultures inhabiting their distributional range. Some

of these animals could have escaped and been introduced into the local lakes and rivers and could have mixed with the native population. Escapees could have great importance for conservation. On the one hand, escaped animals could increase the local genetic variability, which would be important if host populations have been reduced and suffer from inbreeding depression. However, escaped animals can homogenize genetic structuring, possibly leading to a breakdown of local adaptations (outbreeding depression). Before intentionally translocating or releasing any animals in the future, it is important to take into account the genetic characteristics and population sizes of the local populations (Edmans 2007).

Acknowledgments We thank the Posgrado en Ciencias Biologicas from Universidad Nacional Autonoma de Mexico UNAM; Drs. Ella Vazquez and Luis Medrano Gonzalez for their valuable insights into the experimental design and data analysis which greatly improved the quality of this manuscript. Funding was provided by Consejo Nacional de Ciencia y Tecnología (CONACYT) fellowship No. 210994; the Turtle Conservation Fund grants in 2004, 2006 and 2009; Conservation International grants in 2007, and 2009; the Philadelphia Zoo Conservation Program Financial Support for 2005, 2006, 2007, 2008, an award from the Philadelphia Zoo Docent council in 2007 and Elizabeth A. Schreiber and Seabird Research, Inc. We are grateful to Robert C. Fleischer and Nancy Rotzel for funding and laboratory support at the SCBI's Center for Conservation and Evolutionary Genetics. To Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT) for the collection permits: SGPA/DGVS/11385/04; SGPA/DGVS/01789/06 to CITES for permits: MX 23732/05; MX 25219/05; MX29137/06; GT175507; MX42603/08; to the government of Belize for permit No. 3176/08; to the USFWS for permits #MA095827-0; MA119634-0/06; MA139456-0/07; MA194812-0/08; EN/FIS/15/04/08(10)Vol1. To SEMARNAT and Procuraduría Federal de Protección al Ambiente (PROFEPA) in Veracruz, and Tabasco. Thanks to all the people who have helped on different parts of this project; for ideas: Miriam Tsuchiya Jerep, for her important help on running the program BEAST, K. Buhlmann and Peter Paul Van Dijk; special thanks to Richard C. Vogt, who help me consolidate the idea of this project and sharing his expertise on the species. Dr. John Polisar, Carlina Peña, Rene Calderon, Humberto Bahena, Matha Vaca, Raul Rodriguez, Lisette Ramos, Greg Georges, Sam Rivera, Judith Rangel, Erasmo Cazares, Darwin Jimenez, Samuel Cabrera, Kenia Laparra, Manuel Acevedo, Claudia Zenteno, and Filiberto Ascencio Jimenez, the staff from WCS in Flores, Guatemala, all the volunteers, and staff at the turtle farm in Nacajuca, Tabasco, and Zoologico Yumka, Fishermen Cooperative of La Union in Quintana Roo for their help in the collection and measuring of the animals. Thanks also to Basilio Sanchez Luna, Gregorio de la Cruz López, and Sharon Matola for allowing us to work with the animals at their facilities, and to Dr. Jonathan Campbell and Carl Franklin from the Amphibian and Reptile Diversity Research Center of the University of Texas at Arlington for the donation of turtle samples for this study, to Joseph R Mendelson, for the information on Phylogeography of the genus Bufo, and to three anonymous reviewers whose comments greatly enhanced the quality of this manuscript.

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