UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

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Conservación de la Tortuga Blanca *Dermatemys mawii* (Testudíneas: Dermatemydidae)

T E S I S

QUE PARA OBTENER EL GRADO ACADÉMICO DE

DOCTORA EN CIENCIAS

P R E S E N T A

GRACIA PATRICIAGONZALEZPORTER

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P r e s e n t e

Me permito informar a usted que en la reunión ordinaria del Comité Académico del Posgrado en Ciencias Biológicas, celebrada el día 26 de septiembre de 2011, se aprobó el siguiente jurado para el examen de grado de **DOCTORA EN CIENCIAS** de la alumna **GONZÁLEZ PORTER GRACIA PATRICIA** con número de cuenta **87525890** con la tesis titulada: **"CONSERVACIÓN DE LA TORTUGA BLANCA *Dermatemys mawii* (Testudines: Dermatemydidae)"** realizada bajo la dirección de: **DR. OSCAR ALBERTO FLORES VILLELA.**

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Sin otro particular, me es grato enviarle un cordial saludo.

A T E N T A M E N T E
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Resumen:

La Tortuga blanca (*Dermatemys mawii*), es una especie estrictamente acuática y en peligro crítico de extinción. Este proyecto es parte de un programa de conservación y a través del manejo de la especie.

En total se muestrearon 253 individuos de esta especie pertenecientes a 15 localidades a lo largo del área de distribución geográfica de la especie, en México, Guatemala y Belice. El proyecto se dividió en dos partes principales; en la primera se usaron marcadores genéticos de DNA mitocondrial, y en la segunda marcadores nucleares. Para la primera parte se realizó un análisis filogeográfico, para el cual se obtuvieron secuencias de 238 individuos de las 15 localidades de los genes de Cyt*b* y ND4 y se identificaron 16 haplotipos diferentes. En general, nuestros resultados muestran señales de estructura filogeográfica a lo largo de su distribución natural, que aparece opacada por un intenso flujo génico secundario. Este es visible, en la estructura genética a lo largo de las tres cuencas hidrológicas de importancia que actúan como barreras geográficas para taxones acuáticos. Los tiempos de divergencia de los haplotipos de DNA mitocondrial sugieren que los tres linajes genéticos se separaron durante el Plioceno-Pleistoceno hace de 3.73 a 0.227 millones de años y las pruebas demográficas indican que esta especie ha sufrido fluctuaciones demográficas drásticas en tamaño desde este periodo de tiempo. Un haplotipo ancestral (1D) exhibe una divergencia de hasta 2% desde los otros linajes o haplogrupos. Divergencia de esta magnitud es indicativa de nivel de diferenciación de especies en otros géneros de tortugas. El haplotipo 1D sólo fue encontrado en dos localidades, en Sarstún y en Salinas, pero especímenes con otros haplotipos también fueron encontrados en esas mismas localidades. No se sabe si los individuos con haplotipo 1D se encuentran entrecruzándose con ejemplares con otros haplotipos. Nuestros resultados sugieren que la actividad humana, como es la recolecta y transporte hacia lugares alejados podría haber influido en los patrones actuales de diversidad genética. *Dermatemys mawii* ha sido consumida por más de 3000 años, por los habitantes de Mesoamérica, y los registros arqueológicos contienen evidencia fehaciente de que los mayas transportaron animales entre poblaciones hasta lugares alejados de su área de distribución natural. Por lo tanto, los patrones a gran escala de compartir haplotipos hasta entre las barreras hidrológicas existentes, la poca diversidad de haplotipos observada en algunas poblaciones y la ausencia contemporánea de patrones fuertes filogeográficos, es probable que se deba a una combinación de expansión poblacional, flujo génico, movimientos intensivos mediados por seres humanos y cuellos de botella muy recientes, resultados de la sobre-explotación actual de la especie.

En la segunda parte del proyecto se llevó a cabo un estudio usando DNA nuclear de microsatélites y un intrón. Para esta parte se usaron las 253 muestras de las mismas 15 localidades de la primera parte del proyecto. Se diseñaron siete microsatélites polimórficos, y el intrón R35 usado para huellas digitales genéticas. Los resultados de ambos estudios fueron consistentes; en ninguno de los dos juegos de marcadores (mitocondriales y nucleares) se encontró correlación entre distancias genéticas y geográficas. Todos los microsatélites se encontraron en equilibrio de Hardy-Weinberg. No hubo evidencia de desequilibrio gamético para ninguno de los casos. La población de la cuenca del Papaloapan muestra varios alelos únicos, además de tener los niveles menores de flujo génico, que entre las demás poblaciones fue relativamente alto, aún cuando las localidades se encontraran separadas por grandes distancias. Un análisis de estructura bayesiano usando el programa STRUCTURE agrupó a las localidades en dos grupos, uno conteniendo a las localidades situadas al noroeste del area de distribución natural de la especie y otro conteniendo a las del sureste del area de distribución, además de mostrar la singularidad de la población de Papaloapan. No se encontró evidencia de ninguna reducción poblacional reciente, esto puede deberse a que *D. mawii* se considera como una especie longeva, lo cual podría enmascarar estas reducciones. Los resultados de la secuenciación del intrón R35, agruparon a los organismos en tres grupos principales, muy similares a los linajes genéticos encontrados con DNA mitocondrial. El linaje 1D definido con DNA mitocondrial fue separado también con DNA nuclear, tanto de microsatélites como con el intrón. Estos resultados sugieren que posiblemente barreras geográficas como del Istmo de Tehuantepec y la Sierra de Santa Marta han aislado a las poblaciones del Papaloapan del resto de las poblaciones de *D. mawii*. Se propone manejar estas poblaciones como dos ESUs, una para Papaloapan, y otra para Salinas y Sarstún por la presencia del haplotipo 1D y al resto de las poblaciones, como una gran MU, con el fin de preserver la diversidad genética de la especie.

Abstract

The Central American river turtle (*Dermatemys mawii*) is a strictly aquatic species, and it is considered critically endangered by the IUCN, this project is part of a conservation program for this species. 253 tissue samples were collected from 15 localities along the geographic range of distribution of this species, in Mexico Guatemala and Belize. This project was dividen into two main parts. On the first part mitochondrial DNA markers were used, and on the second nuclear markers (microsatellites and one intron). For the MtDNA analysis, fragments of sequences of two genes, Cytb and ND4, were used and sixteen different haplotypes were identified. Results of the MtDNA analysis indicated that a phylogeographic structure did exist throughout the range of distribution; however this structure appears to have been secondarily blurred by extensive gene flow. There was evidence of genetic structuring across three major hydrological basins that also pose bio-geographic breaks for other aquatic taxa. Specifically, divergence times derived from the mtDNA haplotypes in *D. mawii* suggest that the main lineages split during 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 the other haplogroups. Divergence of this magnitude is indicative of species level differentiation in other turtle genera (it is not known whether the individuals with the 1D haplotype interbreed with non-1D individuals). Haplotype 1D was found in only two localities, Sarstun and Salinas; specimens with other haplotypes were also found in those localities.

Analysis 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, geneflow, extensive human-mediated-movements and recent bottlenecks resulting from over-harvesting.

On the Second part of this project, 253 tissue samples were used, from the same 15 localities of the first part. Seven new polymorphic microsatellites were designed, and six of them were used, beside one microsatellite loci designed for another turtle specie, and the fingerprinting

intron R35. Results from the two studies were consistent; neither study found a correlation between genetic and geographic distance.

All microsatellite loci were in Hardy-Weinberg equilibrium. No evidence of linkage disequilibrium in any of the cases was detected. The population from the Papaloapan basin showed high percentage of prívate alleles, and the lowest levels of gene flow from the rest of localities, while the geneflow between most of localities was relatively high, even among those localities separated by long distances.

A structure analysis was implemented in which the uniqueness of Papaloapan was evident. Populations were grouped into two main clusters, one containing the localities situated at the northwest of the geographic range and one containing the southeast localities. It was not found evidence of a recent bottleneck. This could be explained because *D. mawii* is considered as a long living species, and a recent reduction of its populations could be masked by this fact.

Results from sequencing the intron r35 grouped the organisms on three groups, very similar to the genetic lineages found with mitochondrial DNA. Inviduals with haplotype 1D defined with mitochondrial DNA, showed high differentiation using both types of nuclear markers. These results showed that the geographic barrier of the Tehuantepec Isthmus, and Sierra de Santa Marta, has isolated Papaloapan populations from the rest of the *D. mawii* populations. It is proposed to manage these populations as two main ESUs, one for Papaloapan, and one for Salinas and Sarstun that is where the haplotype 1D occurs and the rest managed as a MU, in order to preserve the genetic diversity of this species.

I. Introducción:

a. Antecedentes:

Dermatemys mawii es el único sobreviviente de la familia Dermatemydidae (Hutchison y Bramble, 1981; Iverson y Mittermeier, 1980: Romer, 1956), es la especie de tortuga dulceacuícola de mayor tamaño en México (Ernst, y Barbour, 1989, Vogt, Sin publicar). Esta tortuga es casi completamente acuática, las hembras sólo salen del agua para ovipositar cerca de la orilla. Es una especie totalmente herbívora desde su eclosión hasta adulta (Vogt y Flores Villela, 1992). Se encuentra más activa de noche que es cuando se alimenta de pastos, otras plantas acuáticas y frutos que caen al agua (Álvarez del Toro, et al. 1979, Moll, 1986; 1989).
En general, el carapacho de la especie es bajo, ancho y liso, con una quilla en la región media en las formas juveniles. En algunas hembras adultas este carapacho se hace más alto. El plastrón es robusto, conectado con el carapaho por un puente ancho. El lóbulo posterior del plastrón se encuentra invaginado, sobre todo en los machos.
El carapacho es de color café, gris, negruzco o verde oliva uniforme; el plastrón es blanco, color crema o amarillesco. En algunos casos el plastrón adquiere una coloración café oscura, debido a manchas provenientes del sustrato sobre el que habita. La piel y los escudos pueden variar de color, según el hábitat en el que se desarrollen, pero en general es color café gris o verde oliva. La coloración de la cabeza presenta flancos con manchas oscuras, las hembras adultas tienen el dorso de la cabeza color verde oliva o amarillesca, y los machos presentan una coloración amarilla intensa o hasta anaranjada (Vogt et al. Sin publicar).

Su distribución abarca los estados de Chiapas, Campeche, Tabasco, Veracruz, Quintana Roo, Belice y la costa Atlántica de Guatemala (Campbell, 1998; Smith y Smith, 1979). Mide hasta 65cm de largo del carapacho, y llega a pesar hasta más de 20 Kg (Álvarez del Toro, 1960, Iverson y Mittermeier, 1980, Ernst y Barbour, 1989; Iverson, 1992).

Dermatemys mawii habita en ríos grandes y profundos y sus tributarios, en lagos y lagunas dentro de su área de distribución. Las aguas de estos cuerpos de agua tienen temperaturas de 24 a menos de 30ºC, y prefiere los sustratos blandos (Polisar 1991, 1992). Prefiere las aguas bien oxigenadas, ya que parte de su respiración la lleva a cabo por la laringe (Winokur 1988), lo que le permite permanecer bajo el agua por periodos de tiempo prolongados.

En cuanto a la genética de la especie se sabe que la especie tiene 56 pares de cromosomas, 7 de ellos macrocromosomas metacéntricos o submetacéntricos, 5 telocéntricos o subtelocéntricos y el resto microsomas, no existen cromosomas sexuales, por ser una especie

con determinación sexual por temperatura. No se observan diferencias entre las bandas G de los macrocromosomas. La región de localización de la organización nucleolar es próxima al centrómero. Los cromosomas de esta especie muestran que las tasas de evolución de estos cromosomas es muy lenta, igual que en otras especies de tortugas Cryptodiras, ya que el arreglo y forma de los cromosomas es idéntica a los de la familia Staurotypidae, también considerada como muy primitiva entre las tortugas (Carr et al. 1981).

La mayor amenaza a esta especie se debe a la sobre explotación de sus poblaciones, esto ha llevado a la especie al borde de la extinción y sus poblaciones naturales han sido extirpadas o se encuentran muy reducidas (Vogt et al. 2005).

Esta reducción en las poblaciones de *D. mawii* puede afectar en su diversidad genética, ya que las poblaciones de tamaño pequeño presentan variedad genética limitada, y al reproducirse entre sí esta diversidad genética cada vez resulta más reducida. Lo cual conlleva a la endogamia que tiene como consecuencias, la pérdida de ciertos alelos, y la fijación de otros, que pueden ser deletéreos, por lo que la adecuación de una población tenderá a bajar y será más propensa a adquirir enfermedades, o a no resistir eventos catastróficos o al azar, por lo que se vuelven más proclives a la extinción (Tudge, 1992; Frankham, et al. 2004).

Otro fenómeno que puede afectar a poblaciones pequeñas es la llamada depresión exogámica (Eguiarte y Piñero 1990) ó en inglés "Out-breeding depresión", se da en poblaciones que han sido aisladas de otras por largo tiempo y han empezado a diferenciarse genéticamente de otras por adaptarse a diferentes presiones ambientales (Frankham, et al. 2004). La reproducción entre los individuos de dos poblaciones puede resultar exitosa, pero la adecuación de sus descendientes, puede ser baja. Lo que puede explicar el poco éxito de algunos programas de reproducción en cautiverio, en que se mantienen y reproducen individuos que pertenecen a diferentes subespecies o "linajes genéticos" que no fueron determinadas de antemano.

El empleo de técnicas moleculares como la secuenciación del DNA, puede resolver incertidumbres en cuanto a la especie, población o hasta la identificación de individuos en algún grupo dado de seres vivos; lo que puede tener implicaciones de gran importancia en los proyectos de conservación de especies amenazadas (Frankham, et al. 2002).

En México así como en todos los países donde se distribuye *D. mawii* existen programas para la protección de la especie, pero éstos no han tomado en cuenta identificar y manejar a las diferentes unidades de manejo que pudieran existir dentro de la especie, por lo que resulta crítico el identificar estas unidades, para ayudar a guiar el manejo, monitoreo y otros esfuerzos de conservación y para facilitar la aplicación de leyes para conservar taxones y

sus hábitats. Las Unidades de Manejo (MU), por sus siglas en inglés, son definidas como: poblaciones que se encuentran independientes demográficamente de otras y presentan variaciones adaptativas distintivas. Estas poblaciones, generalmente no presentan independencia evolutiva a largo plazo o fuerte diferenciación adaptativa. La conservación de diferentes poblaciones es crítica para asegurar la persistencia a largo plazo de la especie (Hughes, et al. 1997; Hobbs y Mooney, 1998).

También se han definido a las Unidades de Significancia Evolutiva (ESU), que son aquellas poblaciones o grupos de poblaciones que merecen ser manejados por separado o prioritarios para la conservación por ser altamente distinguibles, genética y ecológicamente (Hughes, et al. 1997; Hobbs y Mooney 1998). Éstas tienes variaciones adaptativas significativas (Ryder, 1986); son distinguibles morfológicamente tienen distribución geográfica delimitada (Dizon, et al. 1992); son poblaciones recíprocamente monofiléticas, o sea que presentan una divergencia significativa en las frecuencias de sus alelos en loci nucleares y mitocondriales (Moritz, 1994), ocupan diferentes nichos cológicos (Arteaga et al. 2011) y carecen de intercambiabilidad (Crandall, et al. 2000).

Existen pocos trabajos sobre la biología, y ecología de *D. mawii* (Campbell, 1998; Smith y Smith, 1979, Álvarez del Toro, 1960, Iverson y Mittermeier, 1980, Ernst y Barbour, 1989; Iverson, 1992, Polisar 1991, 1992, Ernst, y Barbour, 1989, Vogt y Flores Villela, 1992, Vogt et al. 2006, Moll, 1986, 1989, Zenteno et al 2010), y en el campo de la genética de poblaciones o de la genética de la conservación, estos trabajos son inexistentes, estos proyectos serían de gran valor por la importancia, tanto ecológica como cultural de la especie. No se conocen los diferentes linajes génicos de la especie, ni si sus poblaciones presentan estructura genética o patrones de panmixia, tampoco se conoce la dinámicas de flujo génico de esta especie, ni la diferenciación genética o la historia evolutiva de la especie o la demografía de estos animales. No se han detectado las diferentes unidades de manejo o unidades de significancia evolutiva en las que se podría dividir a las poblaciones para su manejo. Esta información resulta de gran importancia para poder tomar decisiones de manejo de la especie, para su conservación y aprovechamiento.

Por lo que resulta crítico el determinar los diferentes linajes genéticos con análisis de DNA para identificar los diferentes haplotipos y genotipos que hay en las poblaciones de *Dermatemys mawii*.

b. Área de Estudio:

El proyecto se llevó a cabo dentro del área de distribución de la especie de la tortuga blanca, en tres cuencas principales: Papaloapan y Coatzacoalcos en Veracruz, y en la Cuenca del

Grijalva-Usumacinta, en Tabasco, Chiapas y Quintana Roo en México, y en el Petén e Itzabal en Guatemala y en Orange Walk y Belize, en Belice. Las localidades de recolecta fueron las siguientes: Río San Agustín en Papaloapan, Nuevo Atlán en Coatzacolacos, Jonuta, Chilapa en Macuspana, Río Salinas y La Unión en Río Hondo, San Pedro, Laguna Yala, Laguna el Peru, Laguna Sacnab, Lago Salpetén, Cerca de Modesto Mendez en Río Sarstun, Big Falls, Río Belice, Sibún, y Laguna New River (Figura 1).

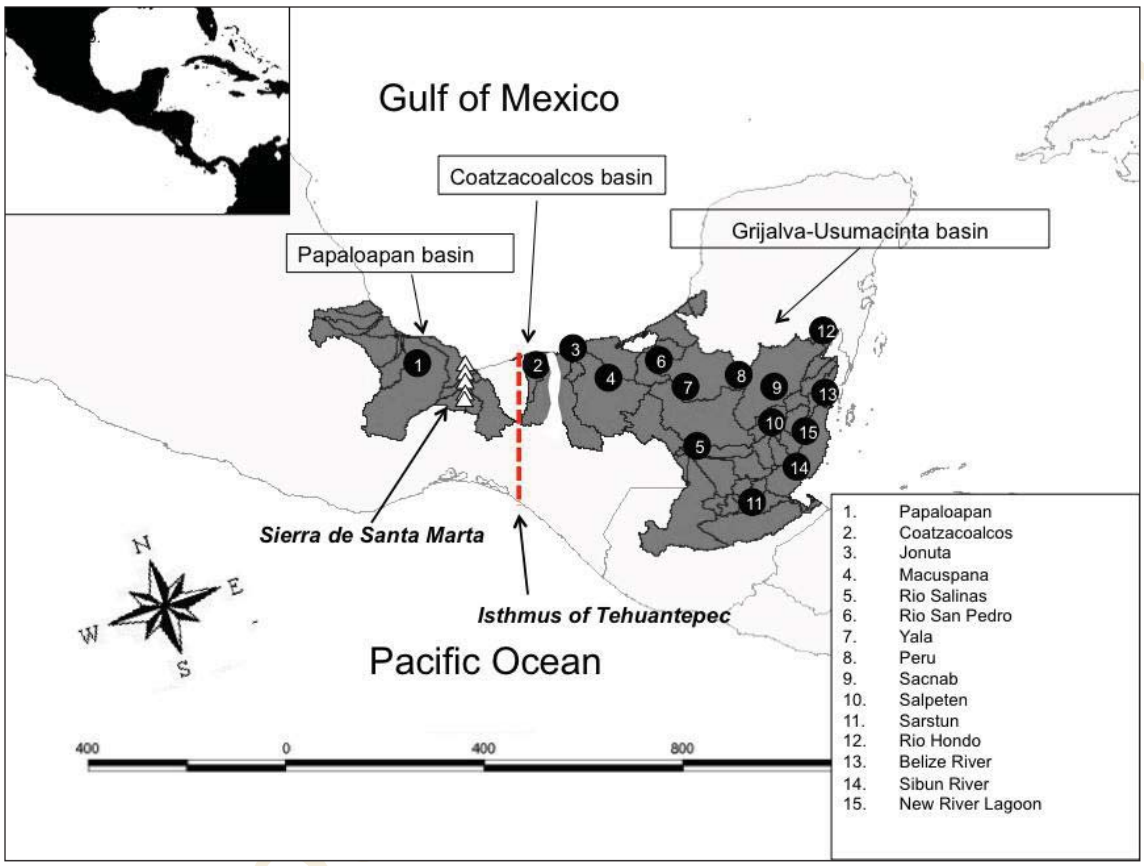

Figura 1. Área de distribución de *Dermatemys mawii,* se muestrán las localidades de colecta y las tres cuencas hidrológicas principales en que se desarrolló el estudio, incluye dos barreras geográficas de gran importancia, el Istmo de Tehuantepec y la Sierra de Santa Marta.

c. Planteamiento Del Problema:

La Tortuga Blanca *Dermatemys mawii* es la especie de tortuga que se encuentra hoy en día en mayor peligro de extinción en México. Ha sido parte de la dieta de los antiguos Mayas y de otros grupos indígenas que han habitado el área de distribución de estas tortugas, siendo considerada como "Manjar de Reyes", pero la comercialización de su carne ha puesto a esta especie al borde de la extinción (Alvarez del Toro et al., 1993; Campbell, 1998). Hoy en día

sus poblaciones se encuentran limitadas a regiones remotas inaccesibles para los seres humanos (Vogt, 1994). Si no se llevan a cabo acciones rápidas y efectivas para su conservación, esta especie corre el riesgo de extinguirse.

En 1980 todavía se podían ver a las tortugas blancas en los mercados de Veracruz; ahora estos animales ya no se venden abiertamente, pues está prohibida su colecta. En Alvarado su carne se vende congelada y sólo por pedido (Vogt, com. pers.). En varias áreas de Belice *Dermatemys mawii* ha sido recolectada tradicionalmente para consumo humano (Moll, 1986; Polisar, 1996). En Guatemala la tortuga blanca es una de las especies de tortuga más valoradas en el Petén, por ser considerada como un manjar (Campbell, 1998). En el pasado era abundante, pero ahora sus poblaciones están muy reducidas debido a su sobreexplotación (Campbell, 1998). En Belice el estado de las poblaciones de esta especie no es claro (Polisar y Horwich, 1994). *Dermatemys mawii* se encuentra categorizada, como en Peligro Crítico de Extinción dentro del libro rojo de la IUCN (Vogt, et al, 2005), en la CITES se encuentra listada en apéndice II (Moll, 1986), CITES, 1994 (http://www.cites.org/esp/app/appendices.shtml), y en la Norma Oficial Mexicana NOM-Ecol-059 (SEMARNAT, 2010) como en peligro de Extinción, por lo que su uso se considera ilegal. Sin embargo a pesar de toda esta protección no se ha podido detener la captura y consumo ilegal de estos animales (Vogt y Flores Villela, 1992).

Existen algunas colonias en cautiverio. Por ejemplo, se sabe que hay más de 800 ejemplares en la granja de Tucta, Tabasco, 20 en el Zoológico Yumka, de Villa Hermosa, Tab., 20 en la "Granja de Tortugas de La Florida" en Veracruz (Aguirre, 2007), además de 4 animales en el Zoológico de Filadelfia y 1 ejemplar el Zoológico de la Ciudad de Guatemala (ISIS 2011: https://www.isis.org/Pages/findanimals.aspx).

Tanto en México como en Belice existen algunas otras poblaciones de *D. mawii* en cautiverio, éstas con un manejo adecuado pueden ser de gran importancia para la conservación de la especie y servir como colonias de aseguramiento "asurance colonies", que en algún futuro pudieran servir para programas de reintroducción de cautiverio. Esto tiene gran importancia cuando se toma en cuenta que en muchos casos el hábitat de esta especie aún no se encuentra perturbado (Ureña Aranda, 2007; Zenteno et al. 2010).

En lo que respecta a este proyecto, se tiene planeado usar la información de genética poblacional y los marcadores desarrollados y probados en este estudio para diseñar un plan de manejo, e implementar colonias de seguridad a lo largo del área de distribución de la especie. Estas colonias serían manejadas genéticamente usando programas como PMx (Lacy y Ballou, 2011), que se basan en identificar las cruzas que mantengan la mayor diversidad genética

posible, incluyendo la cruza con ejemplares de otras poblaciones cautivas también se podría estimar la depresión exogámica y manejar por separado a los diferentes linajes genéticos, por ESUs o por MUs.

Para implementar este proyecto es posible obtener ejemplares con alto valor de conservación (de acuerdo a los resultados de este estudio), como los ejemplares del zoológico de Filadelfia y de granjas de tortugas. Elgunas de estas instituciones ya han manifestado su interés de que sus animales sirvan para la conservación y están dispuestos a que se manejen con estos fines por ejemplo la granja de La Florida en Veracruz, la granja de la Encantada, en Tabasco, el Zoológico de Filadelfia en los EEUU y el zoológico de Chapultepec en la Ciudad de México. Lo anterior se pretende integrar en un proyecto posdoctoral.

En este momento aún no existen las condiciones adecuadas de protección en el hábitat natural para que las poblaciones crezcan, con excepción del proyecto que ya se está llevando a cabo en Laguna el Perú en el Petén, Guatemala, en donde se están incubando nidadas y ya fueron liberadas las primeras crías en esté cuerpo de agua. Pero en el resto de la distribución geográfica es muy importante considerar al manejo en cautiverio como una opción viable para esta especie, ejemplos clásicos de estos proyectos son, el de el Hurón de Patas negras (*Mustela nigripes*) (Miller, et al. 1996) en el Suroeste de los Estados Unidos, o el del Orix árabe (*Oryx leucoryx*)(AAZPA 1987), y en el caso de las tortugas, está el de la tortuga de las Galápagos (*Geochelone nigra*) (Cayot y Morillo, 1997) y el de la tortuga de Mapimí en México (*Gopherus flavomarginatus*) (Aguirre, et al.1997).

El manejo en cautiverio representa un reto ya que para éste se deberán llevar registros confiables (registros de parentesco o “Studbooks” en inglés) que incluyan datos de origen así como particularidades de cada organismo, mantener registros periódicos, como el tener una bitácora del manejo diario de los individuos. Además una buena práctica de crianza en cautiverio que incluya el diseño de una dieta adecuada para cada clase de edad; un monitoreo de la salud de los ejemplares y la correcta manipulación de los mismos, según protocolos ya desarrollados por zoológicos, granjas y centros de conservación de reptiles.

d. Objetivos:

i. **General:**

El objetivo de este proyecto es el caracterizar genéticamente a la tortuga blanca *Dermatemys mawii* y a sus poblaciones con diferentes métodos genéticos, para obtener información que sea de utilidad para proponer estrategias de manejo para su conservación.

ii. **Particulares:**

1. Caracterización genética de la especie, con marcadores genéticos de DNA mitocondrial para poder detectar los diferentes linajes genéticos que pudieran existir.
2. Diseño de marcadores genéticos específicos para *Dermatemys mawii*, de DNA mitocondrial y de microsatélites
3. Se realizarán análisis de de estructura para la especie tanto con marcadores genéticos de DNA mitocondrial y de microsatélites
4. Se identificarán las principales relaciones entre las diferentes poblaciones
5. Se realizará un análisis filogeográfico de la especie.
6. Se identificarán las diferentes unidades de manejo y ESU para proponer acciones de conservación.

e. Estructura del proyecto:

El proyecto se dividió en dos partes principales en las que se utilizaron diferentes estudios genéticos de la especie, la primera parte en la que se trabajó con marcadores de DNA mitocondrial y la segunda en que se trabajó con microsatélites del DNA nuclear.

En la parte correspondiente a DNA mitocondrial se utilizaron dos marcadores: citocromo *b* y ND4, el cual se diseñó en particular para esta especie. Se desarrollaron análisis filogenéticos de la especie y se crearon redes de haplotipos, se hicieron análisis de flujo génico y estructura genética de las poblaciones, se hicieron análisis de divergencia entre las poblaciones y se calculó el tiempo de divergencia entre ellas, se hicieron análisis de neutralidad y de expansión poblacional.

En la parte correspondiente a microsatélites usando DNA nuclear, se diseñaron 6 marcadores genéticos polimórficos y específicos para *D. mawii*. También se probaron 20 marcadores de microsatélites diseñados y usados para otras especies de tortugas, de éstos, sólo uno amplificó y fue polimórfico para esta especie. Con estos siete marcadores se desarrollaron los análisis genéticos correspondientes a diversidad intrapoblacional, estructura genética, ailsamiento por distancia, flujo génico, y análisis de cuellos de botella.

II. Estudio realizado con DNA mitocondrial:

Gonzalez-Porter GP, Hailer F, Flores-Villela OA, Garcia- Anleu R and Maldonado JE (2011) Patterns of genetic diversity in the critically endangered Central American river turtle: human influence since the Mayan age?. Conservation Genetics 12, 1229-1242. DOI 10.1007/s10592-011-0225-x

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RESEARCH ARTICLE

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

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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

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

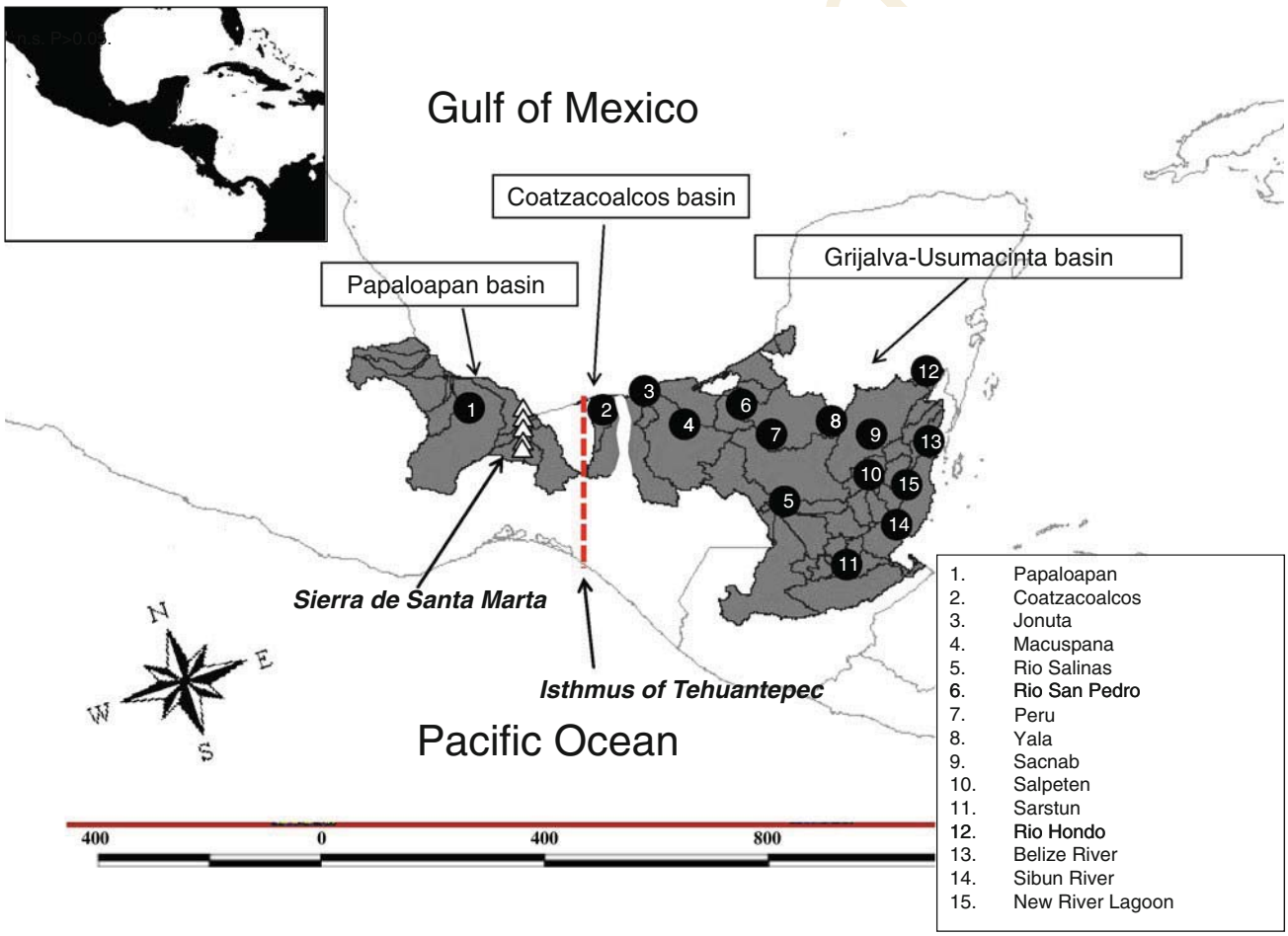

Fig. 1 Geographic distribution of *Dermatemys mawii* (modified from Bulhmann K. 2005). The different main basins are denoted as well as two major barriers, the Isthmus of Tehuantepec and the Sierra de Santa Marta. Sampling localities are: *1*. Papaloapan, *2*. Coatzacoalcos, *3*. Jonuta, *4*. Macuspana, *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

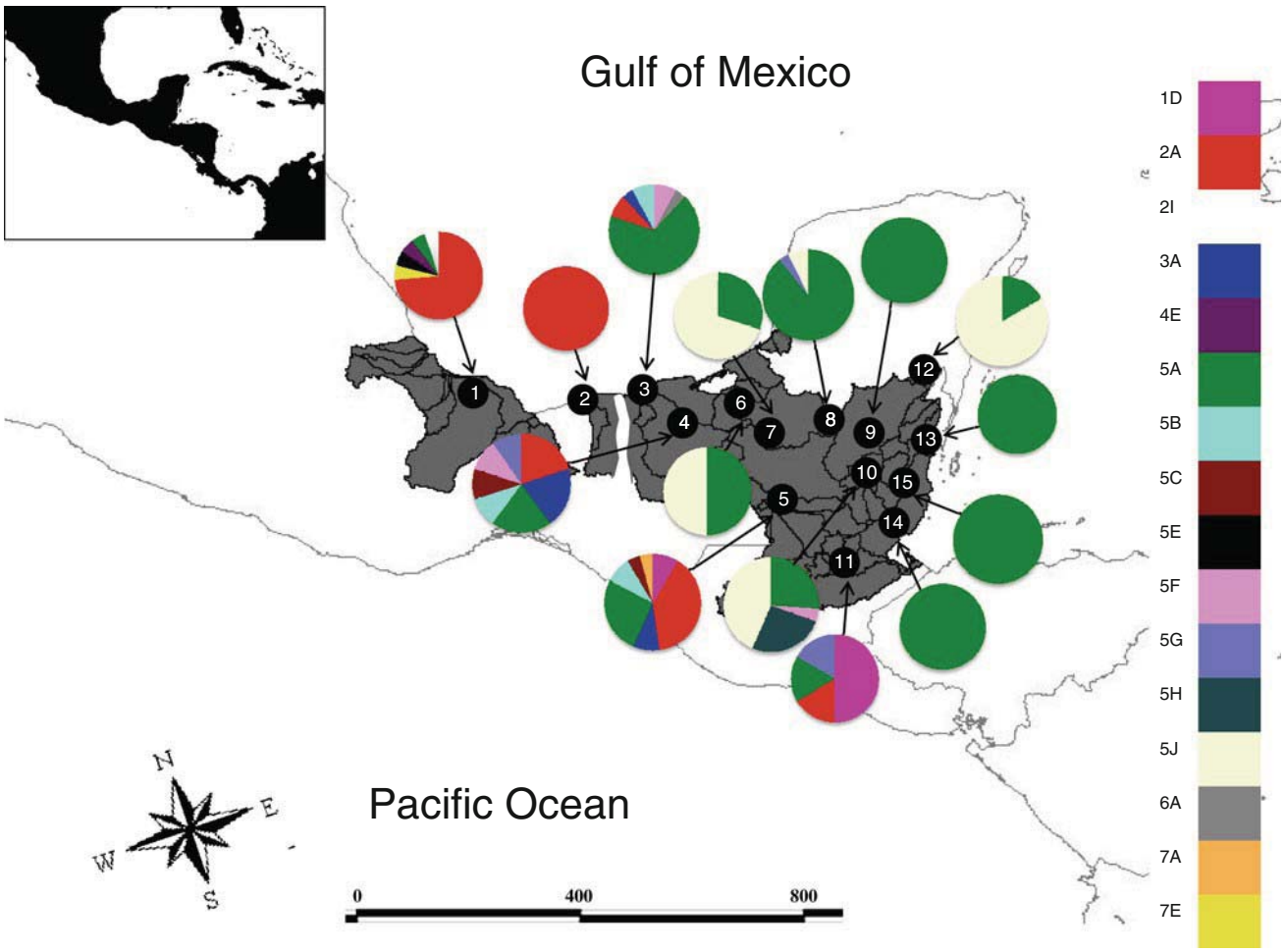

Fig. 2 Haplotype distribution on the geographic range for *Dermatemys mawii*. The *pie graphics* represent the frequencies of haplotypes in each locality

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

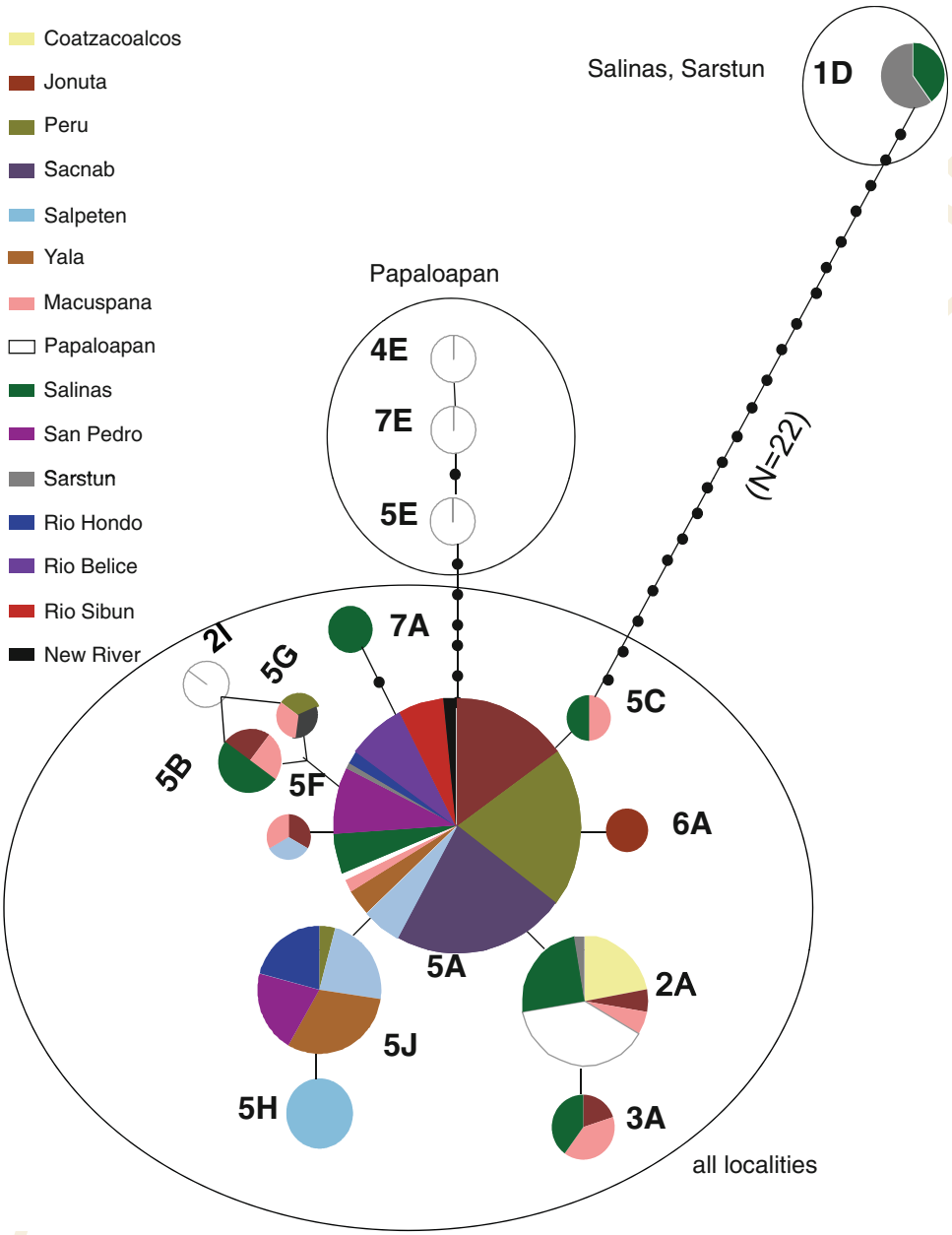

Fig. 3 Statistical parsimony network obtained from TCS (Clement et al. 2000), based on a 95% connection limit. The figure shows three different haplogroups or genetic lineages (Central, 1D, and unique from Papaloapan (PAP), and the localities where these haplogroups occur)

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).

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III. Estudio realizado con marcadores nucleares de microsatélites:

Caracterización genética de la tortuga blanca *Dermatemys mawii* realizada con marcadores nucleares de microsatélites:

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Cryptic lineages and the role of the Isthmus of Tehuantepec as a barrier for gene flow in the critically endangered Central American River Turtle.

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Abstract

We conducted a study of the conservation genetics of the critically endangered Central American river turtle *Dermatemys mawii*, as part of a conservation management program for this species. A previous study identified three distinct genetic lineages based on mitochondrial DNA sequencing of two different markers (Cytb and ND4). The three lineages included a lineage designated PAP, associated with animals found in the Papaloapan River basin in the north of animal's range of distribution, a second lineage designated Central associated with animals found in the Grijalva-Usumacinta Basin

and a third, widely divergent lineage designated 1D found in two localities, Salinas and Sarstun. In this study we used tissue samples from 253 taken from 15 localities throughout the species range of distribution. For this study we developed seven new polymorphic microsatellites, and used one fingerprinting intron R35. Results from the two studies were consistent; neither study found a correlation between genetic and geographic distance.

All microsatellite loci produced 72 alleles, and they were in Hardy-Weinberg equilibrium. We found no evidence of linkage disequilibrium in any of the cases. After rarefaction we found a high number of private alleles in the locality of Papaloapan (64%). Gene flow analysis indicated that the lowest amounts of gene flow were found in the population from Papaloapan ranging from 0.098 to 0.304 and relatively high levels of gene flow between most localities within the Grijalva Usumacinta basin, even those separated by long distances. 7 pairwise localities were non-significant, Macuspana with Salinas, San Pedro with Yala, Peru with Yala, and New River with Belize River, Salinas, Sarstun, and Sibun.

An assignment test present similar results than the gene flow analysis; individuals were in several cases assigned to localities far from the actual point of collection, from 253 samples just 155 were assignment to their collection localities. Isolation by distance analysis revealed no correlation between genetic and geographic distance.

We implemented a structure analysis in which the uniqueness of Papaloapan was evident. Populations were grouped into two main clusters, one containing the localities situated at the northwest of the geographic range and one containing the southeast localities. This result was corroborated using a genetic distance analysis. We found no evidence of a recent bottleneck. This is not surprising since *D. mawii* is a long living species, and a recent reduction of its populations could be masked by this fact. When we analyzed the sequences using the intron R35, we found three main genotypes, one integrated mainly by individuals with haplotype 1D for mtDNA, another one with haplotypes unique to Papaloapan (PAP), and another one with an individual with mtDNA haplotype 2A from Central haplo-group. This result was corroborated when we did a haplotype network, finding these same three groups.

We think the geographic barrier of the Tehuantepec Isthmus, and Sierra de Santa Marta, has isolated Papaloapan populations from the rest of the *D. mawii* populations. We propose to manage these populations as two main ESUs, one of Papaloapan, one of Salinas and Sarstun that is where the haplotype 1D occurs and the rest managed as a MU, in order to preserve the genetic diversity of this species.

Keywords: *Dermatemys mawii*, microsatellites, population genetics, turtle, conservation

Introduction

The Central American River Turtle, *Dermatemys mawii*, is the last surviving species of the giant river turtles of the family Dermatemydidae. This turtle is an important economic and cultural resource for local people throughout its distributional range. Anthropogenic overexploitation (consumption of meat) have caused drastic population declines (Campbell 1998, Polisar 1994, CONABIO et al. 2006), and the species has been listed as critically endangered since 2005 by the IUCN (Vogt et al. 2005), by the US Endangered Species Act (US Fish and Wildlife Service http://ecos.fws.gov/tess_public/SpeciesReport.do), and it is also listed in the Appendix II of CITES (http//www.cites.org/eng/app/appendices.shtml).

The species' distribution range includes parts of Belize, the Atlantic Coast of Guatemala and significant parts of the Mexican States of Veracruz, Tabasco, Campeche, Chiapas and Quintana Roo (Fig. 1). Notably, this area spans across the Isthmus of Tehuantepec and the Sierra de Santa Marta which are major biogeographic breaks in Central America (Croizat 1976) for a variety of bird, mammal, amphibian and butterfly taxa (Duellman, 1960; Peterson et al. 1999; Mulcahy et al. 2006; Rico et al. 2008). In addition, *D. mawii* is distributed along three of the largest watersheds of Mesoamerica, the Papaloapan, the Coatzacoalcos and the Grijalva-Usumacinta river basins (Athie 1987, Hudson et al. 2005).

This study is part of a long-term conservation project to develop conservation management plans by identifying Management Units (MUs sensu Moritz 1999) and Evolutionary Significant Units (ESUs sensu Crandall et al. 2000) for ensuring the long-term persistence of the critically endangered Central American River Turtle. To date, only mtDNA variation in this critically endangered species has been characterized (Gonzalez-Porter et al. 2011); that study found pronounced phylogeographic structure throughout the species range. Most mtDNA haplotypes belonged to a diverse central lineage, and two additional lineage were found in the North-West (Papaloapan drainage basin) and South-East (edge of the Grijalva-Usumacinta basin) at the Salinas and Sarstun rivers, respectively. It was hypothesized that this ancient structuring has been secondarily blurred by extensive gene flow. Notably, this secondary homogenization of genetic structuring also occurred across two major hydrological divides which also cause biogeographic breaks in other aquatic Central American taxa (Peterson et al. 1999; Mulcahy et al. 2006; Rico et al. 2008). Two populations (Sarstun and Salinas) were found to harbor a highly divergent mtDNA haplotype. The individuals carrying this rare and divergent haplotype co-occur with *D. mawii* individuals that carry common haplotypes. Based on mtDNA evidence alone, however, one cannot assess whether members of the different mitochondrial

genetic lineages are capable of interbreeding with each other. Because mtDNA is only maternally inherited, the results of nuclear biparentally inherited markers can be used to determine finer scale population structure, gene flow and genetic variability. Microsatellite markers are commonly used to assess demography, population genetics, phylogeography, estimate population structure, identify the genetic consequences of recent population bottlenecks (Kuo and Janzen 2004; Schwartz and Karl 2005; Pearse et al. 2006), estimate population sizes, migration rates between populations (Nichols and Freeman 2004), and to identify ESUs or MUs (Frankham et al. 2002, Moritz 2002).

From other population genetic surveys in turtles is it clear that nuclear DNA evidence can yield important results not apparent from solely mtDNA. For example *Emys orbicularis* showed low divergence and lack of population structure throughout the Iberian Peninsula using mtDNA, while using nuclear microsatellite loci the results revealed relatively high levels of genetic variability within populations and population structuring (Velo-Anton, et al. 2008). In *Podocnemis expansa* the overall differentiation among populations estimated by F_{ST} with nuclear microsatellite markers was fourfold greater value than the Φ_{ST} values for mtDNA (Pearse, et al. 2006); this could be explained because mitochondrial genes are inherited as a single lineage group or haplotype, and provide only one independent estimate of the species tree and only female inherited. In contrast, a set of nuclear genes can be selected from different chromosomes, such that each gene tree provides an independent estimate of the species tree (Moore 1995). Other explanations could be by female dipersion, or by the different mutation rates of different genes.

A main goal of the present study is to provide information for the conservation and management of the Central American River Turtle by achieving the following objectives: (1) to assess genetic diversity within populations of this species using polymorphic microsatellite loci , (2) to analyze gene flow and genetic structure among populations, defining ESUs and MUs for the optimal genetic management of this species, (3) to assess if *D. mawii* populations have passed through recent bottlenecks, and (4) to compare the patterns observed at microsatellite loci with those recovered from mtDNA in a previous study (Gonzalez-Porter et al. 2011).

Material and methods:

Field and laboratory methods

We collected 253 tissue samples from the hind-foot webbing by cutting small skin snips ($3mm^2$). 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. We avoided cross-individual contamination by using scissors thoroughly cleaned with a 10% bleach solution. All animals were released at the point of collection. The sampling took place between 2004

and 2009 and included much of the current geographic range of this species (Fig. 1). Samples were collected from 15 localities within the following river drainages (Fig 1): from the Papaloapan, and Coatzacoalcos basins, and from the Salinas and Hondo Rivers within the Grijalva and Usumacinta basin in Mexico; from Laguna Peru, Laguna Sacnab, Laguna Salpeten, Laguna Yala, the San Pedro River and the Sarstun River in Guatemala; from the Belize River, New River, and Sibun River in Belize. These samples are from a larger subset of the individuals used in a previous study using mtDNA (Gonzalez-Porter et al. 2011).

Immediately after collection, tissues were preserved in 70% ethanol and later stored in a freezer at -80ºC. We isolated DNA from tissue samples with DNeasy extraction kits (QIAGEN) and proteinase K digestion.

We amplified seven dinucleotide-repeat polymorphic microsatellite loci that we previously designed for *D. mawii* (*Dm3A-32, Dm3A-37, Dm3A-58, Dm3A-13, Dm3A-17, Dm3A-72, Dm3A-42*) (Gonzalez-Porter et al. 2010), and a eighth, GP96, that was designed for *Gopherus polyphemus* but cross-amplifies in other turtle species (Schwartz et al. 2003; Gonzalez-Porter et al. 2010). These markers were fluorescently labeled (HEX or FAM).

DNA was amplified using the following PCR conditions. For Dm3A-32, Dm3A-37, Dm3A-58, Dm3A-13, Dm3A-17, and Dm3A-42, an initial step at 94°C for 7 min was followed by 45 cycles of 92°C for 1 min, annealing at 50-65ºC for 1 min depending on the specific primer pair (50°C for Dm3A-58 and Dm3A-13; 55°C for Dm3A-32, Dm3A-37 and Dm3A-42), extension at 72°C for 1 min, and a final extension at 72°C for 7 min. The conditions for the locus Dm3A-72 were: one initial step at 94°C for 7 min, followed by 38 cycles of 94°C for 40 seconds, annealing at 64ºC for 40 seconds, 72°C for 45 seconds, and a final extension at 72°C for 7 min. The conditions for locus GP-96 (Schwartz et al. 2003) were: 95ºC for 7 min, followed by 38 cycles of 95ºC for 40 s, 50ºC for 30 sec, 72ºC for 1 min, and a final extension at 72ºC for 15 min. The resulting PCR products were analyzed on a 3130XL Genetic Analyzer using Gene scan ROX 500 size standard and genotyped with GeneMapper 4 software (Applied Biosystems).

We also sequenced a novel intron from the RNA fingerprint protein 35 (R35) which has been previously determined to be a single-copy locus that provides excellent resolving power for lineages among turtles (Fujita et al. 2004). We evaluated differences between a subset of individuals with different mtDNA haplotypes, in particular to further elucidate the evolutionary significance of the highly divergent haplotype 1D (Gonzalez-Porter et al. 2011). PCR conditions were as in Fujita et al. (2004): 94ºC for 5 min, followed by 35 cycles of 94ºC for 30 s, 60ºC for 90 sec, 72ºC for 2 min, and a final extension at 72ºC for 10 min. Products were cleaned using Exosap-IT (USB Scientific), cycle

sequenced using BigDye 3.1 (Applied Biosystems) and run on a ABI3130XL instrument (Applied Biosystems).

Statistical analysis:

Genetic diversity within localities

We calculated the mean number of alleles (MNA) per locus, allele frequencies, expected (H_E) and observed (H_O) heterozygosities Nei's (1987), and tested for deviations from Hardy-Weinberg (HW) expectations globally per each population and separately for each locus, using GENEPOP on the web version 4.0.10 (http://genepop.curtin.edu.au/; Raymond & Rousset 1995) by performing the exact test of Guo and Thompson (1992). Sequential Bonferroni correction (Rice 1989) was used to reduce Type I errors for multiple comparisons (α= 0.05). We also used GENEPOP on the web to test for linkage disequilibrium (LD) among loci, and for the exact test we used sequential Bonferroni corrections (Rice 1989). Locus Dm3A-17 was excluded from the study because a significantly lower than expected frequency of heterozygotes was detected, indicating a high incidence of null alleles.

We used the program HP-RARE (Kalinowski 2005) to assess allelic richness (A_R) within localities. HP-RARE uses rarefaction analysis to account for differences in sample size among populations. As a baseline, we standardized sample size to the smallest sample (Sibun, with 7 individuals, i.e. 14 alleles used as baseline sample size) and then determined, using rarefaction, the expected number of alleles in each sample. We also used HP-RARE to determine the private allelic richness (PA; Kalinowski 2004) at each locality.

Due to the low sample sizes for Sarstun (n=5) and New River (n=2), both populations were discarded for rarefaction analysis in HP-RARE. In addition, New River was disregarded in the isolation-by-distance correlations and assignment tests.

Gene flow and Genetic structure

Levels of nuclear DNA differentiation among populations were estimated based on pair wise estimates of the fixation index Θ (Weir and Cockerham 1984) in the program GENETIX 4.05.2 (Belkhir et al. 2000). Assignment tests of individual turtles to specific populations were conducted with GENECLASS 2.0 (Cornuet et al. 1999; Piry et al. 2004), based on Nei's (1997) standard genetic distance. This test computes the composite likelihood of an individual multi-locus genotype to belong to a candidate set of populations, and assigns that individual to the population where the likelihood of its genotype is highest. Isolation by distance was tested by regression of the genetic distance ($F_{ST}/(1-F_{ST})$; Rousset 1997) between pairs of populations against the logarithm of the straight-line geographic

distance measured in kilometers between them. The statistical significance of this correlation was determined by Mantel tests in the Isolation By Distance Web Service IBDWS v 3.16 software (Jensen, et al. 2005).

We used the program STRUCTURE v2.3.1 (Pritchard et al. 2000) to assess levels of genetic structure amongst our sampled localities. This model based (Bayesian) approach performs individual clustering for K distinct clusters,given a prior distribution of the allele frequencies in each population. We explored scenarios with K=1-12 populations using 250,000 iterations as burn-in followed by 500,000 iterations for parameter estimation, based on the admixture model and assuming correlated allele frequencies. Each run was performed 8 times, and results were processed in STRUCTURE HARVESTER v0.56.4 (Earl 2009; http://users.soe.ucsc.edu/~dearl/software/struct_harvest/), which also calculated the ΔK of Evanno et al. (2005). Results of different replicates were combined using CLUMPP v1.1.2 (Jakobsson, Rosenberg 2007). We also constructed a Neighbor-joining (NJ) tree of populations using F_{ST} as a measure of genetic distance, using the program MEGA 4 (Tamura et al. 2007). The resulting grouping was tested with an Analysis of Molecular Variance (AMOVA) included in ARLEQUIN 3.11 (Excoffier et al.1992), to estimate the significance of the geographical arregement.

Demographic tests,

Evidence of a recent bottleneck was assessed using BOTTLENECK v 1.2.02 (Luikart and Cornuet 1998). This program computes for each population sample and for each locus the distribution of the heterozygosity expected from the observed number of alleles (k), given the sample size (n) under the assumption of mutation-drift equilibrium. This distribution was obtained through simulating the coalescent process of n genes under two mutation models, the two phase model (TPM; Di Rienzo et al. 1994), and the stepwise mutation model (SMM; Kimura and Ohta 1978). Because population bottlenecks induce a temporary excess of heterozygosity, finding an observed heterozygosity that is higher than expected heterozygosity for the majority of loci in a population will suggest that this particular population has recently experienced a genetic bottleneck. We used a two-tailed Wilcoxon signed-rank test ($\alpha = 0.05$) (Luikart and Cornuet 1998; Piry et al. 1999). We also tested for mode-shift away from an L-shape distribution of allelic frequencies (Luikart and Cornuet 1998; Piry et al. 1999).

Correlation analysis of mtDNA and Nuclear DNA

In order to analyze the possible concordance between the results of F_{ST} values estimated by (Gonzalez-Porter et al. 2011) with mtDNA, against those estimated using a microsatellite, we conducted a correlation analysis using IBDWS.

To graphically visualize the differentiation between populations, we used the arrangement of the three haplogroups found by Gonzalez-Porter et al. (2011); PAP with haplotypes (4E, 5E, 7E), haplotype 1D and a Central haplogroup containing the rest of haplotypes, based on mtDNA for *D. mawii*. We conducted a Factorial Correspondence Analysis (FCA) using GENETIX 4.05.2 (Belkhir et al. 2004) based on the microsatellite multilocus genotypes.

Results:

Genetic diversity within localities

A total of 253 individuals were genotyped from 15 localities, at 7 polymorphic loci. These 7 loci produced a total of 72 alleles, showing an allelic richness (A_R) average per loci, Rarefied at N14, ranging from 2.1 at Salpeten to 4.5 for Jonuta, a more than 2 fold difference between the minimum and the maximum. Private allelic richness (PA) rarefied ranged from zero in Peru and Yala, to 0.64 in Papaloapan. The observed heterozygosity ranged from 0.329 for Sacnab to 0.609 for Jonuta (mean=0.460) (Table 1). Across all loci, no one locus deviated significantly from HW equilibrium frequencies ($p>0.05$) after sequential Bonferroni correction (Rice 1989),), without Bonferroni correction only the loi Dm3-42 was out of H-W equilibrium with a ($P<0.05$), the rest were in H-W, and the populations, Salinas, Jonuta and Yala were out of H-W equilibrium ($P<0.05$) without Bonferrioni correction, but after this correction only the Salinas samples deviated significantly from HW equilibrium ($P=0.003$) following sequential Bonferroni correction. We did not find any evidence of significant linkage disequilibrium between any pair of loci at any locality ($p>0.05$, applying sequential Bonferroni correction). No locus exhibited large-allele dropout as assessed in MICROCHECKER. Heterozygosities values ranged from 0.33 for Sacnab, and 0.61 for Jonuta. The latter appears to be the most diverse population with the highest levels of allele diversity and heterozygosity, and Sacnab the least diverse population for both allele richness and heterozygosity levels.

Gene flow and Genetic structure

Levels of genetic differentiation using the parameter Θ (Weir and Cockerham 1984) showed low differentiation, between New River and the following populations: Belize River with Θ =-0.069, Salpetén Θ =-0.067, and Yala Θ = 0.017 and large differences between Sacnab and Papaloapan with Θ = 0.304; Salpeten and Papaloapan Θ = 0.254 and Sacnab and Macuspana Θ = 0.227 (Table 2). The population from Papaloapan showed high values of differentiation compared to almost all populations;

these Θ values ranged from 0.098 to 0.304 and also had the largest number of private allelic richness (PA; 0.64).

Consistently, many pairwise Θ values were statistically significant ($p<0.05$; Table 2). 7 pairwise localities were non-significant, Macuspana with Salinas, San Pedro with Yala, Peru with Yala, and New River with Belize River, Salinas, Sarstun, and Sibun.

Standard assignment test results revealed that 155 of 251 (61.8%) of individuals were assigned to in their known collection localities. The sites with the highest percentages of well-assigned individuals were Sibun with 100%, Papaloapan with 91% and Sacnab with 86% (Table 3). The sites with the highest percentages mis-assigned individuals were Yala with 25%, San Pedro, with 28.57%, and Belize River with 44.44%. The locality that showed the highest number (20) of mis-assigned individuals was San Pedro, probably due to its location in the middle of the geographic range of distribution. We found no correlation between genetic and geographic distance across our data set when performing isolation by distance analysis with IBDWS, ($Z= 1.56$, $r= 0.007$, one sided $p=0.515$).

Bayesian clustering in STRUCTURE seemed best explained by a grouping into two cluster ($\Delta K=2$; Evanno et al. 2005). We observed that none of these populations were 100% of one genetic lineage (Fig. 2a-c). Populations located in the north west part of the distribution range showed larger proportion of the "white" cluster, and the ones on the southeast side showed a larger portion of the opposite cluster ("gray").

We analyzed the genetic structure of these populations using the program STRUCTURE v2.3.3, and the statistic of Evanno et al. (2005) had a maximum at $\Delta K=2$ (Fig. 2a,b). The distribution of those two clusters across the geographic range of *D. mawii* (Fig. 2c) indicated that no locality belonged to 100% to one genetic cluster. However, localities in the northwest side of the range of distribution were dominated by one cluster, while localities on the southeast side were dominated by the other. These results are coherent with the results from the geneflow analysis where we found high levels of geneflow between localities among the norwest cluster and the same situation among the localities at the southeast of the range of the species. But there are exceptions, like localitie situated long distance apart with high levels of geneflow estimated by Θ values (Weir & Cockerham 1984), for example between Jonuta and Yala with ($\Theta = 0.05$), Or San Pedro and Jonuta with a value of ($\Theta =0.06$), where one locality is situated at the Norwest cluster and the other on the Southeast cluster.

The NJ tree of populations (Fig. 3) showed two groups, one including populations from Papaloapan, Coatzacoalcos, Jonuta, Macuspana, and Salinas, the other group comprised Salpeten, San

Pedro, Peru, Hondo River, Sacnab, Yala, New River, Sibun, Belize River and Sarstun. This grouping was significant when tested with an AMOVA, ($p(F_{CT})$=<0.001).

Population reduction test

No signal of a recent bottleneck was detected in any of the localities using BOTTLENECK v 1.2.02. No comparison was significant for the Wilcoxon test, and the Mode-shift test for the TPM or SMM models. We removed localities with less than 15 individuals, because this test is size sensitive to sample size and could bias our results (Table 4).We did several repetitions of the test obtaining similar results on each one. We thus concluded that there was no significant signal of any bottlenecks for any of the populations analyzed (p>0.05). These results are not surprising, since *D. mawii* is a long living species, and a recent change could be masked by the long span of generations; this may explain why the Tajima´s test was not significant for expansion using mtDNA genes.

Correlation between mtDNA and Nuclear DNA

Across all populations, patterns of nuclear genetic differentiation are concordant with results obtained using mtDNA from the same 15 populations (Gonzalez-Porter et al. 2011). Pair wise nuclear F_{ST} values were significantly correlated with pair wise mtDNA $\Phi_{ST,mt}$ values from the same populations (using a classic Mantel Test for comparing to matrixes, at, 20000 randomizations, Z=-12.63, r=-0.23, p=0.038) .

Results of a study using mtDNA on *D. mawii* (González-Porter et al. 2011), revealed some highly divergent haplotypes, these were called “Mytotypes” 1D, 4E, 5E and 7E. Haplotype 1D was found in Salinas and Sarstun and these two localities were differentiated from the rest; however, microsatellite analysis of these two localities did not reveal any differentiation. In addition, haplotypes 4E, 5E and 7E were only found in Papaloapan resulting in significant mtDNA differentiation from the rest of the localities. Results using nuclear microsatellites corroborate these findings and also show high levels of differentiation with low levels of gene flow.

In order to visualize the patterns of differentiation between localities we used an FCA. Results of this analysis (Fig 4) clustered the majority of individuals in one big group, including the individuals from Papaloapan but excluding the individuals with mytotype 1D. Two individuals with haplotype 1D were located by the analysis relatively close to the main group, while two (from Salinas) were located well away from the main group. Based upon this analysis, we concluded that in general, individuals with the mitochondrial haplotype 1D are highly divergent from the rest of the individuals.

We also compared sequences from a 930 bp fragment of the nuclear intron R35 from all individuals in our study and found two changes, one at position 786 for two individuals (of mytotype Central), and one at position 922 for five individuals from different mytotypes (Central and PAP) , the rest shared the same genotype. Published sequences for *D. mawii* in Genbank (Acc #1R35Dm:JN655665; 80R35Dm: JN655666; 2R35Dm: JN655667; 3R35Dm: JN655668).

We used the program PHASE included in DNASP 5.01(Rozas et al. 2003). In order to obtain the arragement of these genotype, as a result from this analysis we obtain three genotypes (1, 2 and 3) one with individuals of the mytotype 1D, but also integrating two individuals from the mytotype Central; the second genotype include three individuals of mytotype PAP, and Central, (one individual was incoroprated to two of these genotypes) (Table 5).

Then we performed a TCS analysis too to obtain a haplotype network obtaining results consistent with the tree resuled from the PHASE analysis, with three haplotypes, one including individual of the PAP mytotype, one Including the individuals If mytotype 1D, and one with only one individual from the Central mytotype (Table 5).

Discussion

In this study we used microsatellite loci to look at higher resolution than using mtDNA, patterns of genetic structure in *D. mawii* throughout its distributional range. We were able to detect strong signals of genetic differentiation maybe caused by barriers to gene flow by the Isthmus of Tehuantepec and the Sierra de Santa Marta, also unexpectedly high levels of gene flow amongst populations that are separated by long distances along the Grijalva-Usumacinta basin. These results revealed that individuals were frequently assigned to localities far away from their collection sites, which could be explained by the long distance connection of rivers within the Grijalva-Usumacinta basin may be recently belonging to a just one population. Alternately, it could be the result of human translocation of individuals due to the high economic value of the species; this process may well have been going on for hundreds of even thousands of years (Gonzalez-Porter *el al.* 2011).

Our results support the findings of the previous study using mtDNA data with similar results on the pattern of structure, lacking of correlation between genetic and geographic distances, divergence between all individuals and the ones carrying the haplotype (1D), and levels of gene flow between populations. However the result for the microsatellite´s study showed large genetic differences between Papaloapan and the rest of the localities, as was revealed with high levels of Θ, and on the structure analysis (Fig. 2), (Table 2), and the individuals carrying haplotype 1D were not differentiated from the rest on the Structure analysis (Fig. 2).

Effects of the transition zone of Isthmus of Tehuantepec and the Sierra de Santa Marta upon the different populations:

The analysis of genetic diversity shows that Papaloapan has the highest number of private alleles and the structure analysis confirms that this population is highly divergent from the rest, and the results of the structure analysis using STRUCTURE, all individuals from Papaloapan were grouped as one cluster different than any other one, it is evident from K=3 to K=12 (Fig. 5). Furthermore this population showed the largest Θ values has, what could be translated as, the population reach the equilibrium or as the lowest levels on gene flow of all the populations included in the study therefore these data revealed that this population has been genetically isolated from the rest for long time. The uniqueness of this population could be explained because this is the only population included in this study that is located north-west of the Isthmus of Tehuantepec and the Sierra de Santa Marta (Fig. 1). Several authors (Duellman, 1960; Croizat 1976; Peterson et al. 1999; Mulcahy et al. 2006; Rico et al. 2008) considered the Isthmus of Tehuantepec to be an important biogeographical zone, where major changes in the distributional patterns of many groups occur, considered as a bifurcation zone (Peterson et al. 1999). Geographically it is a very complex zone, where major tectonic events, such as sea level changes and continental uplift, have created small isolated basins within a very large plain. Sea level oscillations during the Miocene to Pleistocene allowed intermittent communication among the hydrological basins within the State of Veracruz (Huidobro et al. 2006, Barber and Klicka 2010, Zarza et al. 2008, Sullivan et al. 1997, Beu 2001). This isolation coincides with the divergence time estimated with mtDNA, for the Pliocene-Pleistocene for the Papaloapan river basin region (González-Porter et al. 2011).

It is interesting to note that most of the populations in this study are located to the south-east of the Isthmus of Tehuantepec in the Grijalva-Usumacinta river basin. This species is highly aquatic and the rivers in this basin have been interconnected for thousands of years and therefore it is not unexpected to find the relatively high levels of gene flow between localities that we observed. However, it is notable to find such high levels between localities that are separated by long geographic distances (more than 400 km) and also the miss-assignment of individuals into populations that are located at considerable distance from their site of collection. Our results also support the fact that anthropomorphic mediated gene flow due to the translocation of individuals to distant areas may have been an important factor that may have influenced the lack of genetic structure in such a large basin. The great economical importance of this species as food and its use for trade or as part of peace offerings to the dominant ruling culture for many Mesoamerican cultures (Gonzalez-Porter et al. 2011) has probably been occurring since the time of the Olmecs, the first pre-Columbian civilization in Mexico *circa* 3000 years ago (Emery 2001, 2005, O'Day *et al.* 2004. Castellano-Cabrera 2007).

Comparison between results from the mtDNA and nuclear DNA results

In general, results from the mtDNA study (Gonzalez-Porter et al. 2011), are concordant with our results obtained with nuclear DNA of *Dermatemys mawii* from the same 15 populations. In both no significant patterns of isolation by distance were found for both genetic markers, and a significant correlation between F_{ST} and Φ_{ST} was found.

The high levels of divergence of the Papaloapan population compared to the rest of the populations are shown in both studies. In the study using mtDNA (González-Porter et al. 2011) we found 3 haplotypes (4E, 5E, and 7E) that are part of the highly divergent genetic lineage that were called "PAP". Individuals with mtDNA haplotype 1D were up to 2% divergent from all the analyzed haplotypes and the genetic structure analysis partitioned the localities containing these individuals into two separate groups (Salinas and Sarstun); however when using nuclear DNA from microsatellites, these differences were not observed, probably due to the small number of individuals that were sampled with haplotype 1D, however the FCA clearly divided all individuals into three groups, one with individuals carrying haplotype 1D, another one with rare haplotypes unique to Papaloapan (4E,5E and 7E), and one with the rest of individuals carrying more common haplotypes. The resulting plot showed a big cluster of all individuals, including those with a unique haplotypes from Papaloapan, and outside from this cluster the individuals with haplotype 1D, two of them located outside of the big cluster, and the other two located far away from it (Fig. 4), showing a high divergence of individuals 1D from the rest also using nuclear DNA. This divergence was also corroborated with the intron R35 revealing that all individuals 1D were grouped as one genotype on a genotype network using this intrón made with TCS and PHASE, we called this genotype "1". This intrón has a slower mutation rate than the mitochondrial DNA markers.

In addition, the rarefaction for private alleles, grouping all individuals into the three genetic lineages or mytotypes showed that the lineage 1D shows high number of private alleles. Showing that the divergence of this group (1D) is ancient, and more studies of this genetic lineage should be done.

Demographic effects on long living organisms

Our result from the population reduction test indicated that none of the *D. mawii* populations analyzed showed significant evidence of recent demographic bottlenecks. Possibly, the long lifespan of *D. mawii* (at least more than 50 years) has had a buffering effect against loss of genetic diversity (see Hailer, et al. 2006). As in numerous other species with a long generation time, significant levels of

genetic diversity may have been retained through demographic fluctuations, allowing to develop conservation actions (Kuo and Janzen 2004).

Conservation recommendations:

González-Porter et al. (2011) in their mtDNA study of this species recommended to consider one ESU for the localities where the haplotype 1D inhabits and one MU for the rest of the localities. and They also recommended to use bi-parental nuclear markers to define other genetic lineages that may deserve special protection; taking into account the results of the present study, we recommended that the Central American River Turtle, *D. mawii'*s population from Papaloapan should be managed as a separated ESU (Crandall et al. 2000: Hughes et al. 1997; Hobbs and Mooney 1998) due to its uniqueness at nuclear and mtDNA level. Also consistently with the mtDNA study (González-Porter et al. 2011) we recommend managing Salinas and Sarstun as another ESU, due to the presence of *D. mawii* individuals carrying haplotype 1D. And the rest of populations should be considered one big MU due to their similarities and because the majority of them live within the Grijalva-Usumacinta basin.

We also recommended to continue studying the genetic lineage 1D, in order to know if genetic isolation occur within the populations where they inhabit and to implement morphometric analyses of these individuals in order to find differences, and finally to perform field studies on the areas where these individuals live to study if there are differences in their natural history.

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Figure captions:

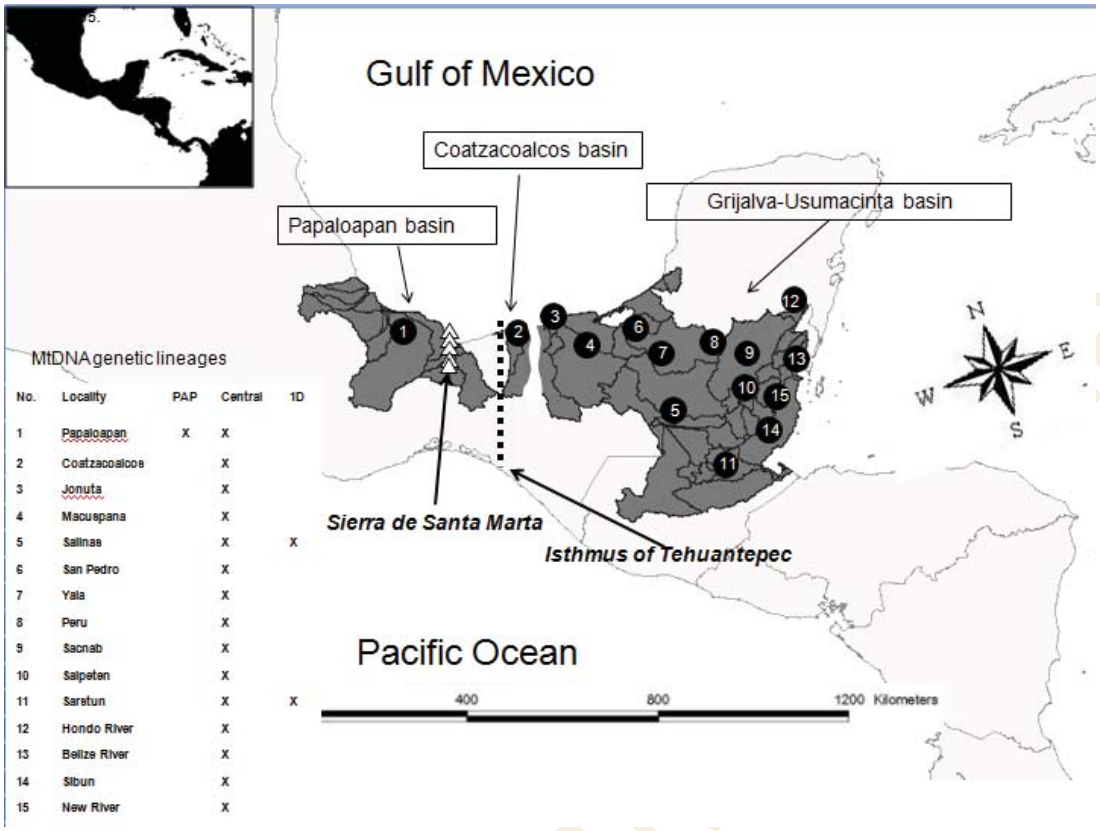
Figure 1. Geographic distribution of *Dermatemys mawii* (dark gray shading; modified from Bulhmann 2005). The main river basins and biogeographic barriers in the region are shown. Numbers 1 to 15 denote the sampling localities and cover almost all of the species' distribution. PAP, "Central" and 1D are the three mtDNA haplogroups,. Dark lines correspond to rivers. Note that Papaloapan is located more than 100 km north of the Isthmus of Tehuantepec.

Figure 2. Structure analysis a) K= 2 populations. b) Plot of K=2 from the Results using STRUCTURE and visualized with CLUMPP, none of the localities show 100% of one group of genotypes, however the populations located on the northwest part of the distribution range showed more percentage of one groups of genotypes, while the populations located at the east part of the range showed highest percentage of the opposite group of genotypes. c). Map of distribution of the different genetic lineages on the different collection localities, represented on pie charts resulted from the structure analysis on STRUCTURE and CLUMPP.

Figure 3: NJ tree based on pairwise genetic distances (F_{ST}) made in MEGA 4, showing two AMOVA-differentiated groups. The first group is located in the north-western part of the range, while the second one prevails in the south-east

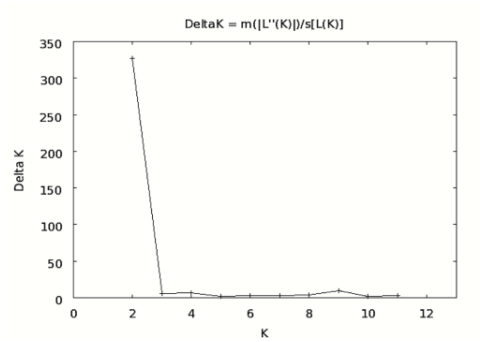
Figure 4: Factorial correspondence analysis (FCA) results for all individuals based on multilocus microsatellite genotypes. Individuals are coded according to their mtDNA haplogroups; PAP (stars), 1D (black dots), and "Central" (white squares). Note that individuals carrying the divergent 1D mtDNA haplogroup also tend to fall outside the main range of nuclear genetic variability.

Figure 5. Plot resulting from the STRUCTURE analysis for K=3, shows the distinctiveness of Papaloapan the only locality situated northwest to the Isthmus of Tehuantepec..

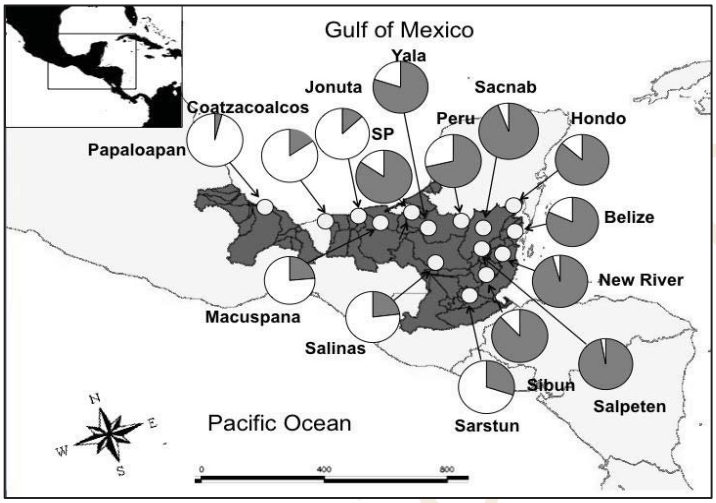


MtDNA genetic lineages

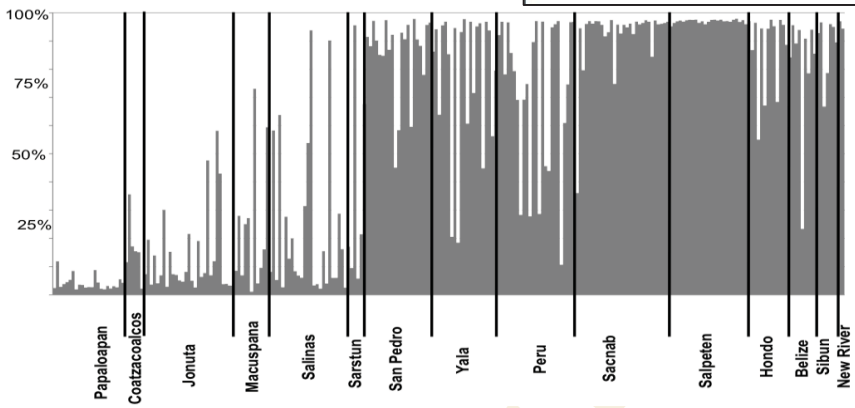
No.	Locality	PAP	Central	ID
1	Papaloapan	X	X	
2	Coatzacoalcos		X	
3	Jonuta		X	
4	Macuspana		X	
5	Salinas		X	X
6	San Pedro		X	
7	Yala		X	
8	Peru		X	
9	Sacnab		X	
10	Salpeten		X	
11	Sarstun		X	X
12	Hondo River		X	
13	Belize River		X	
14	Sibun		X	
15	New River		X	



a

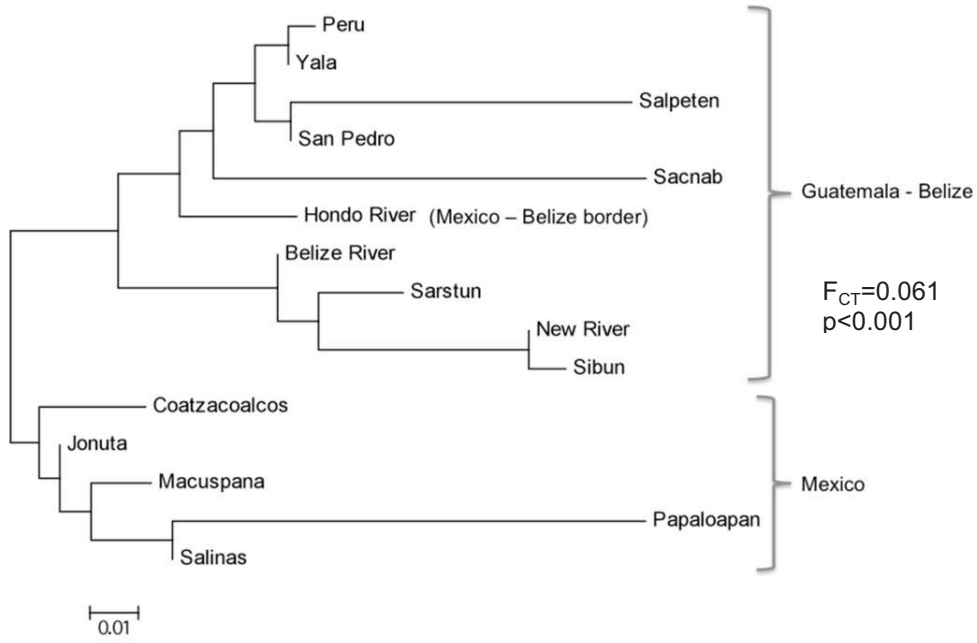


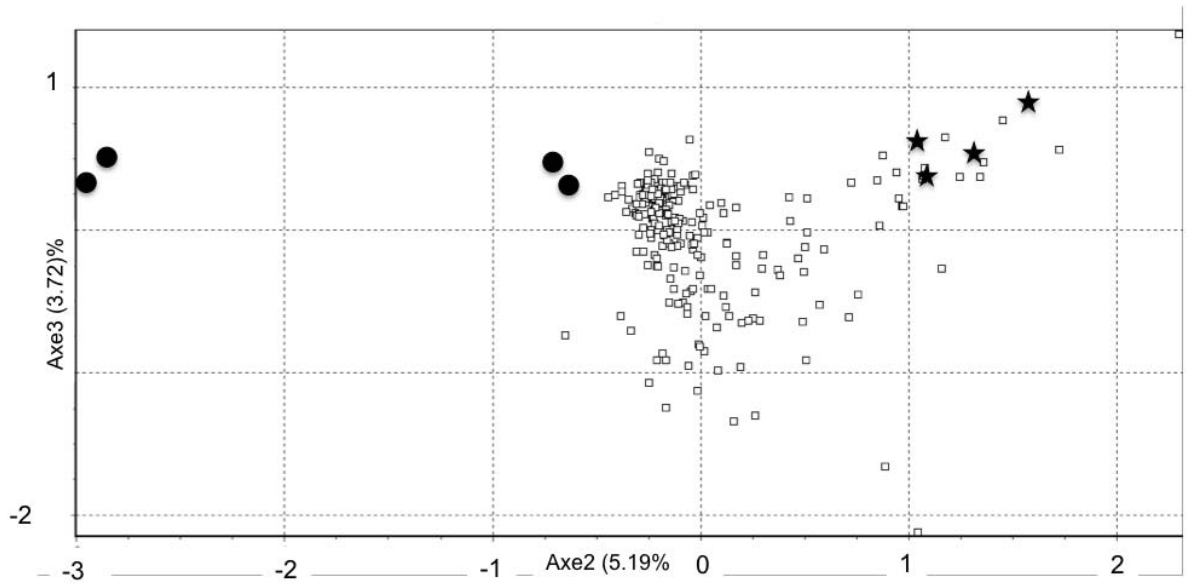
c

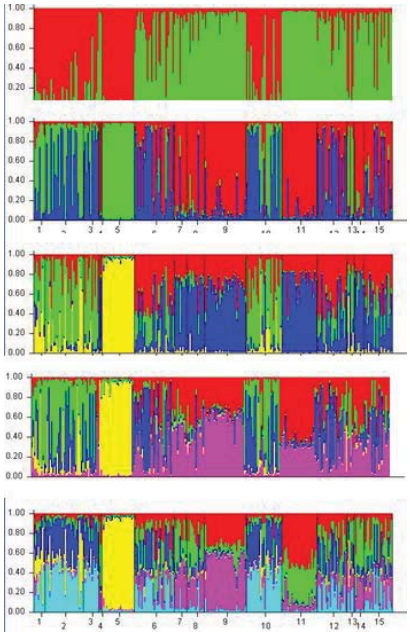


b

NJ







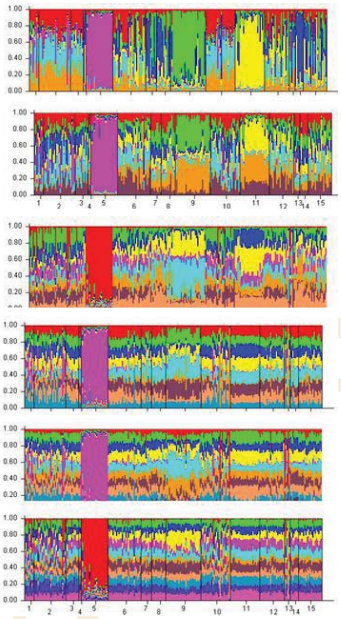
K=2

K=3

K=4

K=5

K=6



K=7

K=8

K=9

K=10

K=11

K=12

Table 1. Genetic diversity within populations. N sample size, H_E and H_O average (multilocus) expected and observed heterozygosity, MNA/locus mean number of alleles per locus, A_R rarefied allelic richness, PA rarefied private allelelic richness. Papaloapan showed the largest number of PA with 0.64, while Yala, and Salpeten have none. Pap=Papaloapan); Coa=Coatzacoalcos; GU=Grijalva-Usumacinta

River basin	Locality	n	H_E	H_O	MNA/LOCUS	A_R (14)	PA (14)
Papaloapan	**Papaloapan**	23	0.522	0.497	5.1	3.8	0.64
Coatzacoalcos	**Coatzacoalcos**	8	0.455	0.482	3.9	3.7	0.22
Grijalva-Usumacinta (GU)	**Jonuta**	27	0.57	0.609	7.1	4.5	0.30
	Macuspana	11	0.531	0.507	5.0	4.2	0.12
	Salinas	25	0.522	0.463*	6.7	4.3	0.29
	San Pedro	21	0.375	0.415	4.1	3.1	0.03
	Yala	20	0.437	0.414	4.3	3.3	0.00
	Peru	28	0.399	0.434	4.1	3.0	0.12
	Sacnab	30	0.317	0.329	3.3	2.4	0.03
	Salpetén	25	0.309	0.343	2.4	2.1	0.00
	Sarstún	5	0.491	0.429	3.1	#	#
	Río Hondo	12	0.457	0.571	3.1	2.9	0.09
	Río Belize	9	0.531	0.556	3.4	3.3	0.03
	Sibún	7	0.436	0.49	2.9	2.9	0.14
	New River	2	0.304	0.357	2.0	#	#

* $p<0.05$; significant heterozygote deficit

Rarefaction and private alleles analyses not performed due to small sample size.

Table 2. Matrix of pairwise genetic differentiation among localities as indicated by Weir & Cockerham's (1984) F_{ST} estimator Θ). The localities are (Pap=Papaloapan, Coa=Coatzacoalcos, Jon=Jonuta, Mac=Macuspana, Sal=Salinas, SP=San pedro, Yal=Yala, Per=Peru, Sac=Sacnab, Salp=Salpeten, Sar=Sarstun, Hon=Hondo River, Bel=Belize River, Sib=Sibun, and NR=New River. These values ranged from -067 on the pairwise New river vs Salpeten, to 0.254 on the pairwise, Papaloapan vs Salpeten. Papaloapan showed high values ranged from 0.098 to 0.254.

Θ	Pap	Coa	Jon	Mac	Sal	SP	Yal	Per	Sac	Salp	Sar	Hon	Bel	Sib	NR
Pap	-	0.121	0.102	0.122	0.098	**0.203**	**0.176**	**0.196**	0.304	**0.254**	0.128	**0.217**	**0.181**	**0.194**	**0.173**
Coa		-	0.020	0.070	0.053	0.090	0.074	0.099	0.231	0.136	0.111	0.1404	0.133	**0.169**	0.142
Jon			-	0.022	0.0184	0.06	0.050	0.074	0.168	0.132	0.084	0.101	0.093	0.122	0.050
Mac				-	**0.003**	0.085	0.068	0.090	**0.227**	0.152	0.079	0.150	0.128	0.145	0.096
Sal					-	0.078	0.064	0.086	0.193	0.142	0.054	0.132	0.098	0.139	**0.073***
SP						-	0.011	0.007	0.081	0.061	0.103	0.049	0.055	0.127	0.022
Yal							-	**0.004***	0.111	0.08	0.075	**0.040***	**0.043***	0.087	**-0.017**
Per								-	0.129	0.089	0.106	0.060	0.077	0.142	0.069
Sac									-	0.188	0.178	0.116	0.084	0.243	0.159
Salp										-	0.211	0.120	0.002	0.058	**-0.067**
Sar											-	0.120	**0.031***	0.070	**0.049***
Hon												-	0.031	0.070	0.049
Bel													-	0.064	-0.069
Sib														-	**0.004***
NR															-

*=Non significant (p>0.05).

Table 3. Results of the assignment test, with known sample origins in rows, and estimated source populations in columns. N is the number of samples from each locality, %self is the self-assignment rate.

Locality*	Pap	Coa	Jon	Mac	Sal	SP	Yal	Per	Sac	Salp	Sar	Hon	Bel	Sib	N	% self
Pap	**21**	2	-	-	-	-	-	-	-	-	-	-	-	-	2	91.3
Coa	-	**6**	2	-	-	-	-	-	-	-	-	-	-	-	8	75.0
Jon	1	-	**16**	3	-	2	-	1	1	-	-	-	-	1	2	59.3
Mac	-	-	1	**6**	-	1	1	2	-	-	-	-	-	-	1	54.6
Sal	1	-	-	2	**14**	2	-	-	-	-	3	2	-	1	2	56.0
SP	-	-	1	-	1	**6**	2	5	2	2	-	1	1	-	2	28.6
Yal	-	-	1	-	-	6	**5**	3	2	-	-	-	2	1	2	25.0
Per	-	-	-	-	2	5	2	**13**	2	1	-	1	1	1	2	46.4
Sac	-	-	-	-	-	1	2	-	**26**	-	-	1	-	-	3	86.7
Salp	-	-	-	-	-	3	-	1	1	**18**	-	-	1	1	2	72.0
Sar	-	-	-	-	-	-	-	-	-	-	**4**	-	-	1	5	80.0
Hon	-	-	-	-	-	-	2	-	1	-	-	**9**	-	-	1	75.0
Bel	-	-	-	-	1	-	-	-	1	-	1	2	**4**	-	9	44.4
Sib	-	-	-	-	-	-	-	-	-	-	-	-	-	**7**	7	100.0

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

Table 4. Population reduction test made with BOTTLENECK, on bold there are three populations that showed shifted curve on Mode Shifted. Overall we find no clear evidence of strong bottlenecks, as it was expected from the long generation length of the species.

Locality	n	TPM	SMM	Mode shift
Papaloapan	23	None	None	Normal
Jonuta	27	None	None	Normal
Salinas	25	None	None	Normal
San Pedro	21	None	None	Normal
Yala	20	None	None	Normal
Peru	28	None	None	Normal
Sacnab	30	None	None	Normal
Salpetén	25	None	None	Normal

Localities with less than 15 individuals were removed from the analysis due the size sensitivity of it

Table 5. Haplotype reconstruction using PHASE with the intrón R35, also include the mtDNA haplotypes, localities where these individuals occurred, and the genetic lineage assigned using mtDNA. Individual 80 as assigned to haplotype 1 and 2.

Haplotype	individuals,	haplotypes mtDNA	Localities	mtDNA lineages
1	Phi4, Phi1,52, 518, 201, 80	1D, 5A	Sar, Sal, Jon	1D, GU
2	80, 325, 87, 111, 13, 118	5A,4E,6A,3A,7A	Jon, Pap, Sal,	GU, PAP
3	1	2A	Sal	GU

IV. **Discusión**

Este es el primer estudio filogeográfico de especies de tortugas dulceacuícolas Mesoamericanas y Centroamericanas. Es también el primer estudio genético de *D. mawii,* única especie viviente de la familia monotípica Dermatemydidae. Se conoce muy poco sobre su biología y ecología de esta especie en peligro crítico de extinción debido al consumo humano, la modificación del hábitat es una amenaza secundaria, en la actualidad existen grandes extensiones de hábitat en buenas condiciones para la especie, pero las poblaciones se encuentran disminuidas o devastadas (Zenteno et al. 2010). En el presente estudio, se implementaron diferentes análisis genéticos para evaluar los niveles de estructura filogeográfica en *D. mawii*, y si existen diferencias significativas entre las cuencas hidrológicas a lo largo del área de distribución.

Análisis Filogenético

Como resultado del análisis filogenético de esta especie se identificaron tres linajes filogenéticos divergentes a través de una red de haplotipos. Uno constituido por el haplotipo 1D, éste es altamente divergente de los otros con divergencias genéticas de hasta un 2%. Divergencia de esta magnitud es indicativa de nivel de especies para otros géneros de tortugas, como por ejemplo *Graptemys* (Lamb et al. 1994) para Citocromo *b* y región control, este ultimo mostrando tasas de mutación parecidas a las de ND4. Los animales con el haplotipo 1D sólo fueron encontrados en las localidades de Sarstún y de Salinas, en estas mismas localidades también se encontraron especímenes con otros haplotipos. Dos linajes genéticos diferentes pueden divergir debido a la existencia de una barrera geográfica existente, pero si esta barrera desaparece, los individuos pueden entrar en contacto y mezclase otra vez. La presencia del linaje ancestral (1D) mezclado con otros haplotipos de otros linajes genéticos puede explicarse por la desaparición de barreras geográficas al flujo génico como resultado de causas naturales o mediadas por acción humana. Los patrones observados pueden ser el resultado de un contacto secundario y un entrecruzamiento subsecuente. Una explicación alternativa, podría ser que esos dos linajes genéticamente aislados no se estuvieran entrecruzando. Pero como el DNA mitocondrial sólo revela a los linajes maternos, no podemos diferenciar entre estas dos hipótesis. Por lo tanto, resultará de gran importancia hacer análisis de parentesco para clarificar los patrones a una escala más fina y determinar si los individuos 1D se encuentran entrecruzándose o no con los otros linajes distribuidos más ampliamente. Los individuos con el haplotipo 1D no muestran diferencias morfológicas aparentes (*Obs.Pers.*), pero se requiere de un estudio morfológico detallado para garantizar la existencia o no de éstas diferencias. Las muestras de Sarstún sólo cuentan con 6 individuos, tres de los cuales tienen el haplotipo 1D. Es importante realizar estudios en esta área

donde éste haplotipo fue encontrado (Sarstún y Salinas), con el fin de buscar otros haplotipos que pudieran explicar mejor la historia de este linaje. Un estudio de patrones de movimientos usando métodos de marcaje y recaptura, pero la realidad es que estas poblaciones se encuentran tan reducidas por el consumo humano, que este tipo de estudios son casi imposibles de realizar.

En la red de haplotipos también se identificó un segundo linaje incluyendo haplotipos únicamente encontrados en Papaloapan (5E, 7E y 4E) referidos como el haplogrupo únicos a Papaloapan o (PAP). Este linaje presenta una divergencia de hasta un 1%, y también cohabita con individuos con otros haplotipos encontrados también en otras localidades. Este podría representar un linaje resultante de un aislamiento histórico (posiblemente pre-humano), aislamiento causado por el Istmo de Tehuantepec, ya que Papaloapan es la única localidad ubicada al norte oeste de esta barrera geográfica (Guevara-Chumacero et al 2010, Rico et al. 2008), y la Sierra de Santa Marta, que es la región más sureña del Eje Neovolcánico Transversal**,** que separa a especies acuáticas de la cuenca del Papaloapan de otras en otras cuencas hidrológicas (González Soriano et al. 1997), de manera similar a lo que ocurre con el género *Bufo,* en que existen diferentes linajes al oeste y este del Istmo de Tehuantepec y de la Sierra de Santa Marta (Mulcahy et al. 2006). Aunque especies Centroamericanas que habitan tierras bajas aparentemente homogéneas, pueden presentar estructura genética poblacional cuando sus movimientos son inhibidos. Por ejemplo, las ranas arborícolas de ojos rojos (*Agalychnis callidryas*) Centroamericanas muestran una estructura genética pronunciada no solamente cruzando las cordilleras montañosas, pero también las a lo largo de los bosques costeros del Caribe y del Pacífico (Robertson and Zamudio 2009). Por otro lado, las especies con especificidad a hábitats bajos que, por tanto tienen gran capacidad de movimiento, muestran menor estructuración: la tortuga caja de Coahuila (*Terrapene coahuila*) es una especie semi-acuática capaz de moverse por pasos en las diferentes pozas de la región y exhibir altos niveles de flujo génico (Howeth et al. 2008). Y aunque *D. mawii* podría exhibir los mismos patrones de flujo génico dentro de las diferentes cuencas, no se espera que existan estos patrones al comparar las poblaciones que se encuentran separadas por altas montañas y hábitats no adecuados para una especie estrictamente acuática. En resumen, lo encontrado para el haplogrupo PAP de cruzar diferentes cuencas hidrológicas y regiones biogeográficas es lo esperado, dados los requerimientos de hábitat estrictamente acuáticos de *D. mawii*.

El tercer haplogrupo, que en el estudio fue llamado Central, incluye a todos los haplotipos restantes y muestra una divergencia considerable dentro del mismo linaje. Los animales con esos haplotipos se encontraron en todas las localidades del área de estudios (por lo tanto incluyendo las localidades con los haplotipos de los otros linajes genéticos; Salinas, Sarstún y Papaloapan). El arreglo de estos haplotipos tiene forma de estrella en la red de haplotipos, lo que es señal de una expansión (de acuerdo con la F_s de Fu). El patrón general del arreglo de los haplotipos incluye una forma de

estrella y dos brazos largos, un patrón similar fue encontrado en cocodrilos de río (*Crocodylus moreletii)*, especie que como *Dermatemys mawii* también ampliamente explotada por humanos (Ray et al. 2004) pero que a diferencia de la red de *D. mawii*, la red de cocodrilos sólo poseía un brazo largo, mostrando más de 20 cambios desde el haplotipo central; este clado central presenta una forma de estrella con de uno a cuatro cambios desde este haplotipo central; el brazo largo mostraba también otro clado en forma de estrella con de uno a cuatro cambios entre sus haplotipos. Diferencias con *C. moreletii* incluyen; la variación genética está de *C. moreletti* está bien estructurada y coincide con el modelo de aislamiento por distancia y *D. mawii* carece de una estructura genética clara. Además, *C. moreletii* no es una especie restringida al agua, siendo capaz de desplazarse por tierra, contrariamente a *D. mawii* que es una especie restringida a los ambientes acuáticos. Esto podría explicar los altos niveles de flujo génico observados entre las diferentes regiones en *C. moreletii*.

La mayor diversidad de haplotipos está concentrada en la parte noroeste del área de distribución geográfica de *D. mawii* (Tabla 1; Fig. 2 estudio realizado con DNA mitocondrial). Las localidades occidentales (como Jonuta, Papaloapan, Macuspana y Salinas) contenían hasta 6 ó 7 haplotipos, mientras que la mayoría de las localidades del sureste del área de distribución sólo poseían uno o dos haplotipos. Algunos haplotipos, como el 2A y 3A, son comunes en la región noroeste del área de distribución pero están ausentes en la región sureste. Lo contrario ocurre con el haplotipo 5J, el cual es común en la parte sureste, pero ausente en el noroeste. Existiendo como un gradiente este-oeste, este resultado fue sorpresivo dado, que el clima en toda el área de distribución de *D. mawii* es relativamente homogéneo. La región se localiza en las tierras bajas del Golfo de México y el Caribe, caracterizada por lluvias abundantes y una temporada de secas relativamente corta y altas temperaturas todo el año (West 1964). Se estimaron indirectamente altos niveles de flujo génico dentro de los diferentes cuencas, conforme a lo predicho y se observó que todas las poblaciones del sureste con excepción de Salpetén y de Sarstún, comparten la mayoría de su diversidad haplotípica. En particular, el haplotipo 5A y 5J, los cuales se encuentran separados por una única sustitución, y fueron encontrados en por lo menos el 96% de los individuos de esta región.

Historia Evolutiva de D. mawii

De acuerdo con Savage (1966, 1982), el género *Dermatemys* ya existía en México y Centroamérica desde hace 55 millones de años durante el Eoceno; sin embargo no existen fósiles del género o la familia en Mesoamérica (Carrol 1988; Flores-Villela 1993; Reynoso 2005). El estimado de la divergencia de secuencias entre los linajes va de 0.227 a 0.752 millones de años si se usa una tasa de divergencia rápida de 2%, y de 1.125 a 3.73 millones de años si se usa una tasa de divergencia lenta de 0.4% (Avise et al. 1992). Sin tomar en cuenta las grandes diferencias entre estas dos tasas de

mutación, se puede concluir que la media de la divergencia intra-específica en *D. mawii* ocurrió durante el Pleistoceno y/o el Plioceno (Tabla 3 del estudio realizado con DNA mitocondrial).

La época del Plioceno-Pleistoceno se caracterizó por cambios climáticos extremos, con periodos glaciales e inter-glaciales y con periodos más fríos y secos que durante el presente (Duellman 1966; Leyden 1984). Durante el Plioceno el clima de Centroamérica se caracterizó por ser un periodo de clima seco y frío (Stuart 1957, 1966). Durante ese tiempo, *D. mawii* pudo haber existido en el Estado de Veracruz, (Savage 1966), después de migrar desde Norteamérica, a través de las tierras bajas. Durante el Pleistoceno, los niveles marinos fueron de más de 100 m más bajos que los niveles actuales, y esto se debió a un patrón de lluvias menores a las actuales. Por esta razon, los niveles de agua de algunos cuerpos de agua como Laguna Salpetén eran mucho menores a los actuales y solamente se llenaba de agua durante la temporada de lluvias (Hodell et al. 2008). Las áreas que actualmente se localizan dentro de las mismas cuencas hidrológicas puede que no hayan estado conectadas por agua por carecer de ésta durante los periodos secos del Pleistoceno, resultando en una diferenciación genética. Las condiciones climáticas fluctuantes durante el Plioceno-Pleistoceno, con periodos de alternancia de humedad y sequedad (Lee 1980), pudieron haber impactado en gran medida a la estructura genética de las poblaciones. Bajo estas condiciones, las poblaciones de esta especie de tortuga altamente acuática, que no puede moverse en tierra, pudieron haberse aislado en pequeños cuerpos de agua remanentes durante los periodos secos (Campbell 1998). Cuando el clima cambió volviéndose más húmedo y los cuerpos de agua se expandieron, las inundaciones pudieron haber permitido el crecimiento poblacional y facilitando el movimiento entre los bajos de los ríos permitiendo a las tortugas dispersarse largas distancias. El flujo génico entre las diferentes cuencas hidrológicas pudo haber ocurrido mediante agua salobre, o hasta por agua salada ya que *D. mawii* se ha observado ocasionalmente que entra a aguas salobres o saladas en Laguna de Términos, Campeche, en Bahía de Chetumal, Quintana Roo y en Sibún en Belice (P.G.G.-P., *obser. Per.*, Feb, 2009). Un mecanismo de dispersión similar se ha sugerido para tortugas del género *Graptemys*, las cuales también son altamente acuáticas y cuyas hembras adultas también hacen sus nidos a pocos metros de los cuerpos de agua en donde habitan (Lamb et al. 1994).

Las condiciones climáticas y geológicas en la cuenca del Grijalva-Usumacinta, que cubre aproximadamente la mitad del área de distribución natural de la especie, eran muy parecidas durante el Pleistoceno a las que prevalecen hoy en día (Leyden 1984), aún cuando se sabe que eran más secas y frías que en el presente. En algunas sequías severas, algunos lagos de esta región (p.ej. Salpetén) se secaban por gran parte del año. Las condiciones climáticas de sequías pueden haber causado que algunas poblaciones pasaran por cuellos de botellas demográficos severos causando extinciones, los cuales pudieron haber resultado en drásticas reducciones del número de haplotipos, como lo

observado en Sacnab y en Yala o recientemente fueron colonizadas por algunos pocos organismos co una diversidad genética rediucida (Fig. 2 estudio realizado con DNA mitocondrial). La Cuenca del Grijalva-Usumacinta es la única parte del área de distribución natural de la especie que cuenta con información sobre las condiciones climáticas durante el Pleistoceno, sin embargo podemos anticipar que las otras cuencas tenían condiciones similares.

Falta de estructura poblacional y posibles influencias de grupos humanos, tanto ancestrales como modernos

Aunque el muestreo de este estudio cubrió tres cuencas hidrológicas las cuales se encuentran separadas por hábitat no apto para la especie (cadenas montañosas con alturas de más de 1,000 m, como es la Sierra de Santa Marta), se encontró sólo una estructura geográfica débil y una extensiva mezcla de haplotipos entre la mayoría de las poblaciones de *D. mawii*. Los resultados del presente estudio exhiben una sorpresiva falta de patrones filogeográficos entre las localidades y entre las diferentes cuencas separadas por grandes distancias, y bajo nivel de flujo génico entre las localidades cercanas entre sí con una falta de soporte estadístico para cualquier patrón de aislamiento por distancia. Una posible explicación de estos patrones mezclados podrían ser que hayan ocurrido múltiples colonizaciones (Mulcahy et al. 2006) en los tiempos en que los cuerpos de agua se encontraban conectados, menos probable, que estas tortugas estrictamente acuáticas hayan recorrido grandes distancias, cruzando barreras terrestres, incluyendo montañas, para legar las localidades actuales. Otra hipótesis podría incluir el trasporte de estas tortugas por humanos a lo largo de grandes distancias para su consumo, venta o propósitos rituales. Los individuos con haplotipos comunes podrían haber sido capturados y dispersados. Sin embargo sigue sin esclarecerse, por que los linajes del haplotipo 1D y Papaloapan no fueron transportados para ocupar un área de distribución mayor hoy en día. Esto puede estar relacionado al origen del comercio/transporte en la región donde el linaje Central prevaleció históricamente, o eventos demográficos recientes han enmascarado el transporte mediado por humanos de los otros linajes. Está bien documentado el consumo de la especie por humanos por varios siglos o incluso milenios, ya que es posible que esta especie formara parte de la dieta de la cultura Olmeca hace más de 3000 años (Soustelle 2003). Muchas especies de tortugas alrededor del mundo han sido apreciadas por su valor como fuente de proteína animal; ya que son relativamente fácil de alimentar y de criar en cautiverio, éstas pueden mantenerse vivas en pequeñas pozas en áreas remotas donde no se cuenta con refrigeración (Jenzen y Das 2008). De hecho las tortugas han tenido una gran importancia como fuente de proteína animal para los habitante de las zonas bajas de Mesoamérica desde antes de que los europeos pudieran documentar estas prácticas en esta zona (Ximenez 1967). Los Mayas transportaban animales a diferentes lugares ya sea para fines

ceremoniales o como alimento (Lee 1996). Por lo que no es de sorprenderse que, *D. mawii* fuera una fuente muy importante de proteínas para los antiguos Mayas del Petén (Periodo preclásico 800-400 A.C.). Existen varias referencias de la presencia de restos de estas tortugas (huesos, conchas) en los templos Mayas de Uaxactun (Stuart 1958), Tikal, Petexbatun, Las Pacayas (O'Day et al,. 2004) y San José, Petén, (Emery 2001; Castellanos-Cabrera 2007). Restos de estas tortugas fueron encontrados en Copan en Honduras (Emery 2005), y en Veracruz (Wing 1976 en Iverson y Mittermeier, 1980). Los restos de tortugas eran parte de las ofrendas de entierros de personajes de alta importancia, los cuales se han encontrado dentro del área de distribución actual de la especie (Lee 1996). También se encontró una referencia a un espécimen de *D. mawii* en un entierro en el sitio arqueológico de Teotihuacán, a más de 300 Km del área de distribución natural de la especie (Elson y Mowbray, 2005). Además, de una escultura de esta especie exhibida en la sala Mexica del Museo Nacional de Antropología e Historia y restos de la especie en el Museo del Templo Mayor de la Ciudad de México, descubiertos en la Cuenca de México a más de 350Km de la localidad más cercana dentro del área de distribución conocida de la especie.

Estas prácticas de mantener tortugas en cautiverio para su eventual consumo siguen dándose hoy en día. En Guatemala, individuos de *D. mawii* son mantenidos en pozas de tamaño mediano de traspatio, llamadas "aguadas" en donde las tortugas son fácilmente capturadas cuando sean requeridas (Campbell 1998). Lo mismo ocurre en el Estado de Tabasco, en donde las tortugas son colectadas en su hábitat intencionalmente o no y son mantenidas en estanques rústicos y criadas hasta que ya sean consumidas en alguna ocasión especial o para su venta, en la que pueden alcanzar altos precios. Un Kg de carne de *D. mawii* puede venderse hasta por $1000 pesos (Vogt *com. pers.*).

Se conocen casos recientes de escapes accidentales o liberaciones de estas tortugas (durante inundaciones) a las aguas de los cuerpos de agua cercanos disponibles; por ejemplo algunos individuos escaparon de granjas en Tabasco, durante las inundaciones de 2007 (Semarnat Tabasco, *com. pers.*). No es improbable que esto también ocurriera en los tiempos de los antiguos Mayas. Los patrones observados en *D. mawii* son similares a los reportados para las terrapenes (*Malaclemys terrapin)*. Esta especie habita esteros a lo largo de la costa Este de los Estados Unidos y se ha observado que carece de una estructura poblacional aún cuando esta especie es altamente filopátrica. Este patrón se ha atribuido a la extensiva translocación de ejemplares a principios del siglo 20 para restaurar las poblaciones disminuidas proveyendo con animales criados en granjas por la gran demanda para el mercado de mascotas de esta especie (Hauswaldt y Glenn 2005). Los movimientos artificiales de *D. mawii*, llevados a cabo por cientos o miles de años, en combinación con los cuellos de botella causados por la sobre explotación, pueden explicar los raros patrones de distribución de los haplotipos que se observan en esta especie.

Estudio llevado a cabo con DNA nuclear

En la Segunda parte del proyecto se llevó a cabo un estudio con DNA nuclear de microsatélites y un intrón, para las mismas 15 localidades usando 253 muestras de *D. mawii.* En este trabajo se analizaron los patrones de estructura genética a lo largo del área de distribución de la especie a una escala más fina que en la primera parte de este proyecto, con lo que se pudo detectar una gran señal de diferenciación genética causada por barreras al flujo génico por el Istmo de Tehuantepec y la Sierra de Santa Marta, e inesperadamente altos niveles de flujo génico entre poblaciones separadas a grandes distancias entre sí a lo largo de la cuenca hidrológica del Grijalva-Usumacinta. Estos resultados revelaron que los individuos fueron asignados frecuentemente a localidades lejanas a los sitios de colecta, lo que puede ser explicado por las conexiones a grandes distancias entre los río dentro de la cuenca del Grijalva-Usumacinta. Una explicación alternativa, podría ser el resultado de translocación de individuos por humanos debido al alto valor económico de esta especie por cientos o miles de años (González-Porter *el al.* 2011). Lo que también confirma lo encontrado en la parte de DNA mitocondrial, encontrando resultados similares en los patrones de estructura, falta de correlación entre distancia genética y geográfica, divergencia entre todos los individuos con el linaje genético 1D, y niveles parecidos de flujo génico entre las diferentes poblaciones. Sin embargo los resultados del estudio con microsatélites muestran grandes diferencias entre Papaloapan y el resto de las localidades, como muestran los resultados con altos niveles de Θ, lo que significa bajos niveles de flujo génico, y en el análisis de estructura (Fig. 2, Tabla 2, estudio realizado con marcadores nucleares de microsatélites), y en donde los individuos 1D no se diferencian del resto en el análisis de estructura (Fig. 2 estudio realizado con marcadores nucleares de microsatélites).

Efectos de la zona de transición del Istmo de Tehuantepec y de la Sierra de Santa Marta sobre las diferentes poblaciones:

El análisis de diversidad genética muestra que Papaloapan tiene el mayor número de alelos únicos y el análisis de estructura confirma que esta población es altamente divergente del resto, y que, y los resultados del análisis de estructura realizados usando STRUCTURE, muestran que todos los individuos de Papaloapan se agrupan en clado diferente a todos los demás, lo que es evidente en los plots desde K=3 hasta K=12 (Fig. 5 estudio realizado con marcadores nucleares de microsatélites). Además esta población muestra los valores más altos de Θ, lo que puede traducirse como los niveles más bajos de flujo génico de todas las poblaciones incluidas en el estudio por lo tanto estos datos revelan que Papaloapan ha estado aislada genéticamente del resto por largo tiempo, por lo que se necesitan hacer más estudios con mayor cantidad de muestras para corroborar esto. La singularidad de

esta población puede explicarse por que es la única localidad del estudio situada al noroeste del Istmo de Tehuantepec y de la Sierra de Santa Marta (Fig. 1). Varios autores (Duellman 1960; Croizat 1976; Peterson et al. 1999; Mulcahy et al. 2006; Rico et al. 2008) consideran al Istmo de Tehuantepec como una zona biogeográfica de gran importancia, donde muchos cambios en los patrones de distribución han ocurrido para muchos grupos, es considerada como una zona de bifurcación (Peterson et al. 1999). Geográficamente es una zona muy compleja, donde eventos tectónicos de importancia han ocurrido, como cambios en el nivel del mar y levantamientos de la placa continental, los cuales han creado pequeñas cuencas aisladas dentro de un área baja muy grande. Las oscilaciones en el nivel del mar durante el periodo del Mioceno al Pleistoceno permitieron una comunicación intermitente entre las cuencas hidrológicas en el Estado de Veracruz (Huidobro et al. 2006, Barber y Klicka 2010, Zarza et al. 2008, Sullivan et al. 1997, Beu 2001). Este aislamiento coincide con los tiempos de divergencia estimados con DNA mitocondrial, para el Plioceno-Pleistoceno para la región de la cuenca del Papaloapan (González-Porter et al. 2011).

Es interesante hacer notar que la mayoría de las poblaciones de este estudio se localizan al sureste del Istmo de Tehuantepec en la cuenca del Grijalva-Usumacinta. Esta especie se encuentra restringida a ambientes acuáticos y los ríos en esta cuenca han estdo interconectados por miles de años y por lo tanto no es de sorprenderse el encontrar niveles relativamente altos de flujo génico entre las localidades observadas. Sin embargo, es notable encontrar estos altos niveles entre localidades separadas por grandes distancias geográficas (más de 400 Km) y también se asignaron erróneamente individuos en poblaciones ubicadas a distancias considerables de los sitios de colecta. Los resultados también apoyaron el hecho del flujo génico mediado antropomórficamente debido a que la translocación de individuos en largas distancias, como se mencionó en el estudio de DNA mitocondrial, puede haber sido un factor de importancia que haya influenciado la falta de estructura genética en esta gran cuenca hidrológica además de las barreras geográficas que pudieran haber existido y existen para esta especie. Este proceso pudo haberse dado desde el tiempo de los Olmecas, civilización ancestral precolombina de hace alrededor de 3000 años, la especie como ya se mencionó ha tenido gran importancia económica como alimento, para el comercio y para usarla como tributo a las culturas dominantes de Mesoamérica (González-Porter et al. 2011; Emery 2001, 2005, O'Day *et al.* 2004. Castellano-Cabrera 2007).

Comparación entre los resultados de DNA mitocondrial y nuclear

En general, los resultados del estudio de DNA mitocondrial (González-Porter et al. 2011) son concordantes con los resultados obtenidos con DNA nuclear para *Dermatemys mawii* de las mismas

15 localidades. En ambos no se encontraron patrones significativos aislamiento por distancia y se encontró una correlación significativa entre las F_{ST} y la Φ_{ST} para ambos marcadores genéticos.

Los altos niveles de divergencia en Papaloapan comparados con las demás localidades son evidentes en ambos estudios. En el estudio realizado con DNA mitocondrial (González-Porter et al. 2011) se encontraron tres haplotipos únicos a esta localidad (4E, 5E, and 7E) componiendo al linaje genético llamado "PAP". Y en el estudio de microsatélites esta localidad tuvo mayor número de alelos únicos.

Los individuos con haplotipo 1D para DNA mitocondrial mostraron una divergencia de hasta un 2% de los otros haplotipos analizados, pero para el análisis de estructura usando microsatélites las localidades de Salinas y Sarstún no fueron separadas de las demás; probablemente por el pequeño tamaño de muestra, pero cuando se hizo un Análisis de Correspondencia Factorial FCA, los individuos del linaje 1D resultaron claramente separados, quedando fuera de un gran grupo formado del resto de los individuos (Fig. 4 estudio realizado con marcadores nucleares de microsatélites), mostrando que este linaje también presenta gran diferenciación con DNA nuclear. Esta divergencia también fue corroborada usando el intrón R35, revelando que este linaje también se agrupa con este marcador nuclear, al usar PHASE y una red de haplotipos TCS, pero en el mismo grupo también se incluyeron otros dos individuos con diferentes haplotipos para DNA mitocondrial. Este intrón presenta una tasa de mutación más lenta que los marcadores de DNA mitocondrial; lo que puede explicar la presencia de estos individuos en el mismo grupo con otros haplotipos dentro de estos grupos formados con el este intrón.

Además de estos análisis, se llevó a cabo un análisis de rarefacción para este linaje usando microsatélites, y se encontró que los individuos del linaje 1D tenían un alto número de alelos únicos para el pequeño tamaño de muestra. Mostrando que la divergencia de este grupo de individuos es ancestral, y se requiere de hacer más estudios de este linaje.

Demografía de organismos longevos

Los resultados de la prueba de reducción poblacional reciente (cuellos de botella) indicaron que ninguna de las poblaciones analizadas de *D. mawii* mostraba evidencia significativa de haber pasado por algún cuello de botella. Esto probablemente debido a que *D. mawii* se considera una especie longeva (que vive por lo menos 50 años) y esto amortigua los efectos de la pérdida de diversidad genética (Hailer, et al. 2006). Como en muchas otras especies con tiempos de generación largos, los niveles de diversidad genética son retenidos a través de las fluctuaciones demográficas, permitiendo el desarrollo de acciones de conservación (Kuo y Janzen 2004). Aún cuando para DNA mitocondrial el linaje Central mostró señales de expansión con la F_S de Fu.

Implicaciones de conservación

Las poblaciones de *D. mawii* están en grave riesgo de desaparecer a lo largo de toda su área de distribución natural (Vogt et al. 2005). Aunque existe aún bastante hábitat en buenas condiciones a lo largo de una buena porción su distribución geográfica natural, las poblaciones remanentes han pasado por reducciones demográficas severas debido a la colecta ilegal de sus ejemplares (Vogt, sin publicar; CONABIO-DGVS-CONANP 2006). Por lo tanto, todas las poblaciones de *D. mawii* requieren de una protección especial. Los programas de manejo en cautiverio que mantengan altos estándares de prácticas de crianza, con buenos registros de sus ejemplares y el manejo genético de los mismos deben ser promovidos, para mantener poblaciones genéticamente viables para eventuales proyectos de reintroducción de ejemplares en un hábitat protegido dentro del área de distribución histórica de la especie (Syed et al. 2007).

Nuestros resultados muestran que existen tres linajes genéticos (haplogrupos) de *D. mawii* (Central, 1D y PAP). Es importante mantener poblaciones viables de animales de estos tres linajes e implementar el manejo genético efectivo de estos organismos. Los esfuerzos en cautiverio que se están llevando hoy en día en granjas como la de la "La Florida" y el centro de tortugas en cautiverio con fines de conservación propuesto para la "La Popotera", ambos en Veracruz, necesitan ser apoyados. Y finalmente, es crucial proteger a las poblaciones silvestres de esta especie de las diferentes cuencas hidrológicas a través de esfuerzos de educación ambiental, y a través del apoyo de las autoridades locales (Aguirre, 2007).

Estos tres linajes pueden ser considerados como unidades de manejo, pero como estos organismos comparten el hábitat con individuos de otros linajes, es importante manejar todas las poblaciones de *D. mawii*, en especiel las que presentan linajes altamente divergentes, como son las de Salinas y Sarstún por la presencia del linaje 1D y las poblaciones del Papaloapan, por sus haplotipos y alelos únicos. También es de gran importancia proteger de manera especiel las poblaciones con gran diversidad genética como son las de la región Noroeste del área de distribución de la especie, en especiel, las poblaciones de Salinas y de Macuspana y Sarstún, por la gran diversidad de haplotipos y a la de Jonuta, Salinas, Papaloapan y Macuspana el alto número de alelos únicos.

Otra manera de la diversidad genética de la especie sería mediante el uso de unidades de manejo y de Unidades de Significancia Evolutiva, manejando a la población del Papaloapan como otra ESU (Crandall et al. 2000: Hughes et al. 1997; Hobbs and Mooney 1998) (PAP), por presentar varios haplotipos únicos con DNA mitocondrial (González-Porter et al. 2011), y gran número de alelos únicos y gran diferenciación en el análisis de estructura, usando DNA nuclear de microsatélites. Por lo que el manejo de estos organismos deberá de hacerse por separado a los otros grupos.

También se recomienda que a las poblaciones de Sarstún y Salinas sean manejadas como una ESU para proteger a los ejemplares 1D, por ser altamente divergentes de los otros grupos, hasta con un 2% con DNA mitocondrial, poseer gran nivel de diferenciación con DNA nuclear y gran número de alelos únicos, considerándose un linaje ancestral. Además se recomienda hacer más estudios; morfológicos para ver patrones de variación morfológica a lo largo del área de distribución natural de la especie y de conducta de este grupo para determinar si estos organismos 1D representan un linaje aislado reproductivamente. Por lo que se recomienda llevar a cabo experimentos de reproducción para evaluar la viabilidad de la descendencia. Realizar estos experimentos es factible, ya que varios de estos organismos se encuentran en cautiverio en el Zoológico de Filadelfia y en la granja de tortugas de Tucta, Tabasco.

El resto de los organismos, pertenecientes al linaje "Central" deberán ser manejados como una gran unidad de manejo o MU debido a sus similitudes y a que la mayoría de sus individuos habitan en la cuenca del Grijalva-Usumacinta, pero tomando en cuenta la protección de poblaciones de esta especie que habitan en Jonuta y Macuspana, por poseer gran cantidad de alelos únicos.

Antes de translocar animales en eventuales reintroducciones se deberán de tomar en cuenta las características genéticas y los tamaños poblacionales de las poblaciones silvestres en al ambiente en que se liberarán los organismos, tomando en cuenta todos los puntos que marca la ley para los proyectos de reintroducción (Edmans et al 2007).

V. Conclusiones generales:

1. Usando DNA mitocondrial se encontraron 16 haplotipos diferentes en 15 localidades situadas a lo largo de la distribución natural de la Tortuga blanca.
2. *Dermatemys mawii* presenta tres haplogrupos mitocondriales o linajes genéticos principales: uno con haplotipos únicos a la cuenca del Papaloapan (PAP), otro linaje compuesto por un sólo haplotipo altamente divergente (1D) que habita en los Ríos Sarstún y Salinas, y un tercer grupo que habita en todas las localidades pero, principalmente en las de la cuenca del Grijalva Usumacinta (Central), esto como resultado de una red de haplotipos con DNA mitocondrial.
3. Los tiempos de divergencias entre los linajes genéticos 1D, PAP y Central, son del periodo Plioceno-Pleistoceno, por lo tanto se infiere que los cambios climáticos ocurridos en estas épocas (más fríos y secos, además de cambios en el nivel del mar en a zona del Istmo de Tehuantepec), influyeron en los patrones filogeográficos observados.
4. *Dermatemys mawi* es una especie que sigue en términso generales un el modelo de panmixia, para DNA mitocondrial, aún cuando existe una diferenciación pobre entre sus poblaciones
5. La diversidad genética mayor fue encontrada en la parte noroccidental de la distribución natural de la especie.
6. Se diseñaron siete loci nuevos de microsatélites nucleares para la especie y un juego de primers para amplificar el gen mitocondrial ND4, específicos para *Dermatemys mawii.*
7. No se encontraron evidencias de reducciones poblacionales recientes para la especie.
8. El análisis de estructura usando microsatélites mostró que existe una estructura clara para la especie en K=2, aunque ninguna localidad tiene el 100% de un sólo grupo de genotipos de cada localidad, lo que coincide con los resultados del estudio de DNA mitocondrial de mezcla de haplotipos en cada localidad.
9. Se propone que la mezcla de linajes genéticos en casi todas las localidades puede deberse a la translocación humana, debido a la importancia económica de la especie por varios milenios.
10. El haplotipo mitocondrial 1D es altamente divergente para el DNA mitocondrial, para los microsatélites y el intrón nuclear r35.
11. Papaloapan es una localidad con haplotipos únicos, de acuerdo a los resultados del estudio de DNA mitocondrial y que presenta un alto número de alelos únicos de acuerdo a los resultados del estudio de microsatélites, además tener los niveles más bajos de flujo génico y mantenerse como un grupo independiente en el análisis de estructura, lo que puede deberse a ser la única localidad situada al noroeste del Istmo de Tehuantepec y la Sierra de Santa Marta, barreras bio-geograficas conocidas para gran número de taxa.

12. Ya que *Dermatemys mawii* es una especie en peligro crítico de extinción, es de muy importante manejar los diferentes linajes por separado para asegurar su protección, por lo que se propone que esta especie sea manejada como una gran unidad de manejo (MU). Es importante que las poblaciones que habitan en la Cuenca del Río Papaloapan sean manejadas como una ESU separada por contener haplotipos únicos y divergentes con alto nivel de aislamiento genético; así como otra ESU para las poblaciones que habitan en los Ríos Salinas y Sarstún, ya que en ellas existen organismos con el haplotipo 1D altamente divergente tanto para DNA mitocondrial y nuclear de microsatélites e intrón, ya que estos organismos requieren de una protección más amplia, por encontrarse restringidas a estas localidades y por lo tanto encontrarse en mayor grado de amenaza que el resto de la especie. Por lo que se reqiuieren más estudios genómicos, de adaptación, ecología y fisiología sobre este linaje.

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VII. Anexos:

MOLECULAR ECOLOGY
RESOURCES
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PERMANENT GENETIC RESOURCES NOTE

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Abstract

This article documents the addition of 396 microsatellite marker loci to the Molecular Ecology Resources Database. Loci were developed for the following species: ***Anthocidaris crassispina, Aphis glycines, Argyrosomus regius, Astrocaryum sciophilum, Dasypus novemcinctus, Delomys sublineatus, Dermatemys mawii, Fundulus heteroclitus, Homalaspis plana, Jumellea rossii, Khaya senegalensis, Mugil cephalus, Neoceratitis cyanescens, Phalacrocorax aristotelis, Phytophthora infestans, Piper cordulatum, Pterocarpus indicus, Rana dalmatina, Rosa pulverulenta, Saxifraga oppositifolia, Scomber colias, Semecarpus kathalekanensis, Stichopus monotuberculatus, Striga hermonthica, Tarentola boettgeri*** **and** ***Thermophis baileyi.*** **These loci were cross-tested on the following species:** ***Aphis gossypii, Sooretamys angouya, Euryoryzomys***

russatus, Fundulus notatus, Fundulus olivaceus, Fundulus catenatus, Fundulus majalis, Jumellea fragrans, Jumellea triquetra Jumellea recta, Jumellea stenophylla, Liza richardsonii, Piper marginatum, Piper aequale, Piper darienensis, Piper dilatatum, Rana temporaria, Rana iberica, Rana pyrenaica, Semecarpus anacardium, Semecarpus auriculata, Semecarpus travancorica, Spondias acuminata, Holigarna grahamii, Holigarna beddomii, Mangifera indica, Anacardium occidentale, Tarentola delalandii, Tarentola caboverdianus **and** ***Thermophis zhaoermii.***

This article documents the addition of 396 microsatellite marker loci to the Molecular Ecology Resources Database. Table 1 contains information on the focal species, the number of loci developed, any other species the loci were tested in and the accession numbers for the loci in both the Molecular Ecology Resources Database and GenBank. The authors responsible for each set of loci are listed in the final column. A full description of the development protocol for the loci presented here can be found on the Molecular Ecology Resources Database (http://tomato.biol.trinity.edu/).

Table 1 Information on the focal species, the number of loci developed, any other species the loci were tested in and the accession numbers for the loci in both the Molecular Ecology Resources Database and GenBank. The authors responsible for each set of loci are listed in the final column

Species	No. of primers developed	Other species tested	MER database no.	GenBank accession no.	Authors
Anthocidaris crassispina	11	n/a	44091–44101	HM045499–HM045509	Ng, Wai-Chuen; Chak, Solomon T.C.; Wai, T.C.; Chan, M.N.; Leung, Kenneth M.Y; Leung, F. C. C.
Aphis glycines	10	*A. gossypii*	44039–44048	GU556974–GU556983	Kim, Hyojoong; Kim, Min-Young; Kim, Kyung Seok; Lee, Hang; Hoelmer, Kim A.; Lee, Seunghwan
Argyrosomus regius	10	n/a	43852–43861	GU724789–GU724792, GU724794–GU724799	Porta, D.; Porta, J. M.; Porta, J.; Andree, K.; Duncan, N.
Astrocaryum sciophilum	11	n/a	44216–44226	HM055514–HM055523, EU151465	Girod, C.; Tollon-Cordet, C.; Pfunder, M.; Sarrazin, E.; Samadi, S.; Boisselier-Dubayle, M. C.; Lambourdiere, J.; Riéra, B.; Leblois, R.; Sarthou, C.; Charles-Dominique, P.; Fréville, H.
Dasypus novemcinctus	9	n/a	44081–44090	AC156764*, AC145507†, CH512092, CH512179, CH482426,	Chinchilla, Leah; Woodard, Anastasia; Loughry, W. J.; Brooks, Christopher P.; Welch, Mark E.

Table 1 Continued

Species	No. of primers developed	Other species tested	MER database no.	GenBank accession no.	Authors
				CH482433, CH483824, CH484724, CH484035	
Delomys sublineatus	14	*Sooretamys angouya, Euryoryzomys russatus*	44173–44187	HM036723 HM036736	Sommer, Simone; Taubert, Ramona; Schmidt, Anke; Axtner, Jan; Lieckfeldt, Dietmar
Dermatemys mawii	8	n/a	44192–44199	HM208158, HM208160–HM208163, HM208165, HM208166, AF546888	González-Porter, Gracia P.; Flores Villela, Oscar; Hailer, Frank; Bozarth, Christine A.; Maldonado, Jesús E.
Fundulus heteroclitus	108	*F. notatus, F. olivaceus, F. catenatus, F. majalis*	43873–43924, 43935–43990	AF082696, AY791459, AY791469, AY791484, AY791490, CN953105, CN953671, CN953859, CN954710, CN955223, CN956237, CN959642, CN962780, CN969421, CN970366, CN970819, CN971551, CN972805, CN973425, CN973649, CN974954, CN975907, CN975921, CN976892, CN977013, CN979549, CN980692, CN980761, CN980947, CN980953, CN981880, CN981992, CN982025, CN982077, CN982078, CN982085, CN982208, CN982342, CN982387, CN982601, CN983819,	Jackson, S. A.; Wang, R. L.; Whitehead, A.; Roberts, D. A.; Duvernell, D; Nacci, D.; Bagley, M. J.

Table 1 Continued

Species	No. of primers developed	Other species tested	MER database no.	GenBank accession no.	Authors
				CN984412, CN984794, CN985173, CN985425, CN985451, CN985533, CN985559, CN986186, CN986676, CN987016, CN987460, CN987830, CN988090, CN988379, CN989186, CN989692, CN990067, CN990892, CN991593, CV816789, CV816809, CV817822, CV819694, CV819797, CV820076, CV820894, CV821631, CV821635, CV821898, CV822037, CV822135, CV822194, CV822397, CV823325, CV823584, CV823941, CV824092, CV824272, CV824291, CV824906, CV825002, CV825144, CV825291, CV825390, DN951105, DN951765, DN956261, DN956719, DN956985, DN957089, DN957170, DN957227, DR046376,	

Table 1 Continued

Species	No. of primers developed	Other species tested	MER database no.	GenBank accession no.	Authors
				DR046494, DR046611, DR046800, DR047213, DR109362, DR398035, DR398186, DR441274, DR441281, DR441391, DR441490, DR441729, DR441844, DR442006	
Homalaspis plana	10	n/a	44163–44172	HM191694–HM191703	Segovia, N. I.; Gallardo-Escárate, C.; Haye, P. A.
Jumellea rossii	16	*J. fragrans*, *J. triquetra*, *J. recta*, *J. stenophylla*	44200–44215	GU325613–GU325628	Humeau, L.; Dafreville, S.; Da Silva, D.; Rakotoarivelo, F. P.; Pailler, T.; Guérin, F.
Khaya senegalensis	13	n/a	44293–44305	GU903057–GU903069	Li, Chunhong; Fong, Yokking; Hong, Yan
Mugil cephalus	13	*Liza richardsonii*	44102–44114	HM004324–HM004326, HM004328–HM004332, HM004335, HM004343, HM004345, HM004346, HM004348	Shen, K. N.; Chen, C. Y.; Tzeng, W. N.; Chen, J. D.; Knibb, W.; Durand, J. D.
Neoceratitis cyanescens	14	n/a	44142–44152, 44188–44191	GU807480–GU807493	Delatte, H.; Simiand, C.; Risterucci, A.M.; Quilici, S.
Phalacrocorax aristotelis	10	n/a	44132–44141	GU296113 GU296115, GU296117, GU296118, GU296120, GU296122, GU296123, GU296125, GU296127	Barlow, E. J.; Telford, A.; Daunt, F.; Cavers, S.
Phytophthora infestans	8	n/a	44332–44339	See text for details.	Li, Y.; Govers, F.; Mendes, O.; Testa, A.; Jacobsen, E.; Huang, S. W.; van der Lee, T. A. J.

Table 1 Continued

Species	No. of primers developed	Other species tested	MER database no.	GenBank accession no.	Authors
Piper cordulatum	12	*P. marginatum*, *P. aequale*, *P. darienensis*, *P. dilatatum*	43775–43786	HM118524–HM118535	Lasso, Eloisa; Cerón-Souza, Ivania; Bermingham, Eldredge
Pterocarpus indicus	18	n/a	44275–44292	GU903070–GU903087	Li, Chunhong; Sman, Muhammad Hidayat Bin; Fong, Yokking; Hong, Yan
Rana dalmatina	10	*R. temporaria*, *R. iberica*, *R. pyrenaica*	43765–43774	EU139058–EU139064, FJ687621, GU581316, GU581317	Sarasola-Puente, V.; Beebee, T. J. C.; Gosá, A.; Gómez-Moliner, B. J.; Lizana, M.; Madeira, M. J.
Rosa pulverulenta	17	n/a	44115–44131	GU130162–GU130177	Jowkar, A.; Mardi, M.; Kermani, M. J.; Kafi, M.; Pirseyedi, S. M.; Ghaffari, M. R.; Fattahi, R; Zeinolabedini, M.; Mahmoodi, P.
Saxifraga oppositifolia	10	n/a	43925–43934	GU734329–GU734336, GU734338, GU734339	Pietiläinen, M.; Korpelainen, H.
Scomber colias	8	n/a	44267–44274	AB354595–AB354602	Catanese, Gaetano; Funes, Victoria; Perez, Laura; Infante, Carlos
Semecarpus kathalekanensis	10	*S. anacardium*, *S. auriculata*, *S. travancorica*, *Spondias acuminata*, *Holigarna grahamii*, *Holigarna beddomii*, *Mangifera indica*, *Anacardium occidentale*	44071–44080	FJ656103, FJ656105, FJ656107–FJ656114	Ravikanth, G.; Sumangala, R.C.; Naveen Kumar, L.; Ramesha, B.T.; Vasudeva, R.; Ganeshaiah, K. N.; Uma Shaanker, R.
Stichopus monotuberculatus	15	n/a	44306–44320	GU591965–GU591979	Xia, Jianjun; Hu, Chaoqun; Fan, Sigang; Luo, Peng; Zhang, Lvping

Table 1 Continued

Species	No. of primers developed	Other species tested	MER database no.	GenBank accession no.	Authors
Striga hermonthica	12	n/a	44243–44254	FI776044, FI774622, FI775250, FI775982, FI775983, FI775081, FI776123, FI775072, FI774526, FI775577, FI775578, FI775002	Estep, Matt; Van Mourik, Thomas A.; Muth, Peter; Guindo, Diarah; Parzies, Heiko K.; Koita, Ousmane A.; Weltzien, Eva; Bennetzen, Jeffrey L.
Tarentola boettgeri	10	*T. delalandii*, *T. caboverdianus*	44153–44162	HM212426–HM212435	Tejangkura, T.; Brown, R. P.
Thermophis baileyi	9	*T. zhaoermii*	44062–44070	HM537136–HM537144	Hofmann, S.; Dorge, T.; Solhoy, T.; Miehe, G.

*This microsatellite is associated with the *Dasypus novemcinctus* ortholog of human cytotoxic T-lymphocyte antigen-4.
†This microsatellite is associated with the *Dasypus novemcinctus* ortholog of human vitamin D receptor.

Characterization of eight polymorphic microsatellite loci for the critically endangered Central American river turtle, *Dermatemys mawii*.

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Running title: Microsatellites for freshwater turtles

ABSTRACT

Thirteen microsatellite markers were characterized for the critically endangered Central American river turtle, *Dermatemys mawii*. We also screened 20 markers described for *Gopherus polyphemus* and *G. agassizii*. All loci were screened in 30 *D. mawii* individuals from a population of turtles from the Sacnab Lagoon in Guatemala. We found eight polymorphic and five monomorphic loci. All loci had dinucleotide repeats. Allelic diversity in those eight loci ranged from two to six (mean=3.625), with observed heterozygosity ranging from 0.100 to 0.933 (mean=0.408). All polymorphic loci conformed to Hardy-Weinberg expectations and showed no significant gametic disequilibrium.

The Central American river turtle*, Dermatemys mawii,* is critically endangered (Vogt *et al.* 2006). Assurance colonies exist; however, they lack accurate records of individual turtle origin and the degree of genetic differentiation between localities is unknown. Microsatellite markers can provide estimates of genome-wide heterozygosity, which enables studies of heterozygosity-fitness correlations (Allendorf & Luikart 2007; Mc Gaugh *et al.* 2007). In order for the captive population to be a viable source of re-introductions, it is crucial to identify genetically valuable individuals in captivity as well as to characterize patterns of genetic variation and gene flow of wild populations across the range of this species.

In this study, we characterize seven novel polymorphic microsatellite loci in *D. mawii*, and report an additional polymorphic locus originally developed for another turtle species. We created a microsatellite-enriched genomic library following the method of Hamilton *et al* (1999). DNA was isolated from a tissue sample of a *D. mawii* individual from Papaloapan, Veracruz, Mexico using the DNeasy® extraction kit (QIAGEN®) and proteinase K digestion. DNA was digested with *Hae*III, *Xmn*I, and *Nhe*I restriction enzymes (New England Biolabs). The fragments resulting from this process were size-selected to 200-1200 bp, ligated to SNX linkers, hybridized to biotinylated oligos - $(CA)_{10}$ and $(AAAG)_5$ - and separated from unhybridized fragments using magnetic beads (Dynabeads M-280 Streptavidin, Invitrogen). The remaining fragments were polymerase chain reaction (PCR) amplified with SNX (CTAAGGCCTTGCTAGCAGAAGC) primers, ligated into pBluescript II SK + plasmids, and transformed into *Escherichia coli* XL-10 Gold cells (Stratagene).

We picked 75 clone colonies of each oligo and boiled them for 10 minutes at 100ºC. Then we screened the clones by PCR, using the primer T7 (5'-GTAATACGACTCACTATAGGG-3') and the enrichment oligos, running them out on a 2% agarose gel looking for smears rather

than bands (Dearborn *et al*. 2008). The clones which exhibited smears were amplified with T7 and T3 (5'-AATTAACCCTCACTAAAGGG-3') primers. Then their products were tested for smears on an agarose gel. Clones with inserts in the region of 350-900 bp were sequenced using Big Dye v3.1 Terminator cycle sequencing kits (Applied Biosystems) and cleaned via Sephadex G-50 columns (GE Healthcare). Sequences were analyzed using an ABI PRISM 3100 or 3130XL Genetic Analyzer (Applied Biosystems), and aligned by eye using Sequencher® 4.8 (Gene Codes Corporation).

From the CA-enriched library, we screened 75 clones and sequenced 56, yielding 30 clones with more than five dinucleotide repeats (CA or GA). From the tetranucleotide-enriched library, we screened 39 clones and sequenced 23, yielding three clones with tetranucelotide AAAG repeats. From the tetranucleotide CAAA, we screened 38 clones and sequenced 27, yielding one clone with dinucleotide CT repeats. The obtained sequences have been submitted to GenBank (Dm3A-32: HM208165, Dm3A-37: HM208162, Dm3A-58: HM208160, Dm3A-17: HM208163, Dm3A-13: HM208161, Dm3A-72: HM208158, Dm3A-42: HM208166).

We designed primers for 13 loci using Primer3 (*http://frodo.wi.mit.edu/primer3/*). These primers were tested using PCR and products were run on an agarose gel. Following Schuelke (2000), each PCR reaction was performed using a tagged 3-primer setup in 10μl reactions containing 1.3 μl 10x GeneAmp Gold buffer (Applied Biosystems), 1.2 μλ dNTP (2mM), 1μl $MgCl_2$ (25 mM), 1 μl each of labeled and reverse primers (10 μM), 0.1 μl of the forward primer, 0.15μl Amplitaq Gold polymerase (ABI), and 1μl of DNA (approximately 5-50 ng).

PCR conditions were one initial step at 94°C for 7 min, followed by 45 cycles of denaturing at 92°C for 1 min, annealing at 50-65ºC for 1 min depending on the specific primer set (Table 1), extension at 72°C for 1 min, and a final extension at 72°C for 7 min, in a thermal-cycler machine MJ Research Inc. (Bio Rad) model PTC-100.

We also searched for microsatellite markers designed for other turtle species that might cross amplify for *D. mawii*. We tested microsatellites developed for the desert turtle *Gopherus agassizii*, GOA1, GOA2, GOA3, GOA4, GOA4, GOA5, GOA6, GOA7, GOA8, GOA11, GOA12, GOA13, GOA14, GOA17, GOA22 and GOA 23, but none amplified for our species. We also tested microsatellites designed for the gopher tortoise *G. polyphemus,* GP-19, GP30, GP-61 and GP-96. These markers cross amplify in other turtle species (Schwartz *et al*. 2003). All of these markers amplified for *D. mawii*, but only one locus, GP-96, was polymorphic for this species.

PCR conditions for the GP-96 locus were one initial step at 95ºC for 7 min, followed by 38 cycles of denaturing at 95ºC for 40 s, annealing at 50ºC for 30 s, extension at 50ºC for 1 min, and a final extension at 72ºC for 15 min.

We tested all loci for polymorphism on 30 *D. mawii* individuals from Sacnab Lagoon, Guatemala, using the above PCR protocol and found 8 polymorphic and 5 monomorphic loci (Table 1). Microsatellites were analyzed on a 3130XL Genetic Analyzer using GeneScan ROX 500 size standard, and genotyped with GeneMapper 4 software (Applied Biosystems). We used GenePop v1.2 (Raymond & Rousset 1995) to test for gametic disequilibrium and departures from Hardy-Weinberg equilibrium (HWE) expectations.

Designed primers for the monomorphic microsatellite markers also submitted to GenBank are: Dm3A-30 (HM208164), F: GTCGCCCAGTGTTTAAAAGG, R: TGGGAGAGGATCATGGAAAG; Dm3A-43 (HM208157), F: ACCTCCACCCTAAACCCAAC, R: TCATGTCCCATAGACTGACTTGA; Dm3A-61 (HM208159), F: CCACCCTGCACTCACTGG, R: CACATGCAGGTGCACAATTC; Dm4A-11 (HM208167), F:GAGCTGCTGTCTCAGCTTACTG, R:GCCTCTTACTCTACAAAGCAGCA; Dm2A-4 (HM208156), F:GAAGCATAGGGTAGTCAGTTCCA, R:CCCGATTTTTCACACTTGCT. We report those

sequences for possible use of the loci in cross-species amplifications or additional resources of single-copy genomic sequences (see Hefti-Gautschi *et al.* 2009).

No locus deviated significantly from HWE frequencies ($p>0.05$) and there was no evidence of gametic disequilibrium among loci ($p>0.05$, applying sequential Bonferroni correction; Bonferroni 1936). No locus exhibited large-allele dropout when tested in Micro-Checker (van Oosterhout *et al.* 2004). Among the 30 individuals studied, allelic diversity ranged from two to six (mean=3.625), with observed heterozygosity from 0.100 to 0.933 (mean=0.408) (Table 1) (Nei 1972). In summary, we found eight polymorphic loci that should be useful for studies of relatedness and population genetics in the critically endangered *D. mawii*.

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