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Low genetic variability in the endangered Colombian endemic freshwater turtle *Podocnemis lewyana* (Testudines, Podocnemididae)

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Abstract

The Magdalena River Turtle (*Podocnemis lewyana*) is a Colombian endemic species, endangered due to human exploitation and habitat destruction. To date, this species is poorly known ecologically and data on its genetic diversity are lacking. Here we report on the first genetic survey of the species across its distribution range. We obtained mitochondrial DNA sequences (488 bp) of the cytochrome *b* gene from 109 individuals. Samples belong to populations located at several different localities, grouped in five regions, along the four main river basins: Magdalena, Cauca, San Jorge, and Sinú drainages. We found two haplotypes, which differ in only one nucleotide substitution and which are represented with different frequencies in the five geographic regions. These results suggest that *P. lewyana* harbors little genetic variation and is a genetically uniform species, but more variable markers (i.e., microsatellites) should be used to unravel fine-scale phylogeographic structures in this species.

Contents

Introduction

Colombia harbors 25 of the 53 species of continental turtles and tortoises (Testudines) found in the Neotropics, and is thus a biodiversity hotspot of turtles and tortoises (Ceballos-Fonseca, 2002). Due to habitat loss and human exploitation, 14 of these species are threatened to various degrees (Castaño-Mora, 2002) and registered in the IUCN (International Union for Conservation of Nature and Natural Resources) Red List. Freshwater turtles, tortoises, and crocodiles are the most endangered vertebrate groups (Castaño-Mora, 2002). Colombia harbors all six known species of the fresh water turtle genus *Podocnemis* and all of them are threatened (Castaño-Mora, 2002); *Podocnemis erythrocephala* is listed as vulnerable (VU, Castaño-Mora, 2002), *Podocnemis unifilis* and *Podocnemis expansa* are critically endangered (CR) in the Orinoquian region and endangered (EN) in the Amazonian region (Castaño-Mora, 2002), *Podocnemis vogli* is near threatened (NT, Castaño-Mora, 2002), *Podocnemis sextuberculata* is data deficient (DD), but possibly threatened (Castaño-Mora and Medem, 2002), and *Podocnemis lewyana* is endangered (EN, Castaño-Mora and Medem, 2002). The urgency of improved conservation of these turtles is recognized (e.g., Castaño-Mora, 2002; Vanzolini, 2003).

Podocnemis lewyana or Magdalena River turtle (Fig. 1) is a middle sized, Colombian-endemic freshwater turtle from the Magdalena and Sinú river basins (Ernst and Barbour, 1989). The maximum Straight Carapace Length (SCL) registered is 50 cm in females and 36 cm in males (Gallego-García and Castaño-Mora, in press). Local fishing communities used its meat and eggs for consumption for many years. Traditionally, the consumption levels are highest at Easter when eating turtle is a necessity rather than a tradition (Castaño-Mora and Medem, 2002). Easter time coincides with the dry season throughout the turtle's distribution range. During the dry season, female turtles lay eggs on the beaches of the rivers, and therefore can easily be collected by

local people. As the Catholic Church in Colombia forbids the consumption of chicken and beef during Eastertime, and the availability of fish has decreased, the demand for turtle meat and eggs has opened economic opportunities. Furthermore, young turtles are persecuted as people keep them as pets (Castaño-Mora, 1986).

Podocnemis lewyana is legally protected since 1964, and is taken up in the "National Program for The Conservation of Turtles" (Rodríguez *et al.,* 2002), which includes an action plan for its conservation. However, there is little information available on the ecology and demography or on human activities that interfere with this species. Furthermore nowhere in its distribution range does the species occur in a National Park or other protected area.

Fig. 1. Adult male of *Podocnemis lewyana*. Straight Carapace Length (SCL) = 31 cm. Individual number 17, Prado (Tolima), Upper Magdalena River (Region I). Photo: Juan Manuel Vargas-Ramírez.

Due to their often specific habitat requirements (Pritchard and Trebau, 1984; Ernst *et al.,* 1994), slow sexual development, high juvenile mortality (Castaño-Mora *et al.,* 2003; Paez and Bock, 1998), limited dispersal abilities, and human exploitation, some freshwater turtles species (e.g., *Hydromedusa maximiliani*, Souza *et al.,* 2002) are known to be highly vulnerable to habitat-isolation and destruction, small population size, and loss of genetic variation (Souza *et al.,* 2002). Due to the apparently generally low levels of genetic variation and because many species are in need of conservation measures, identification of the genetic population structure of turtles is important in guiding conservation strategies in many taxa (Janzen *et al.,* 1997). Conservation genetic studies on species of the genus *Podocnemis* have only been carried out in the Amazon region. Sites *et al.* (1999) developed specific microsatellite markers for *P. expansa*. Using six microsatellite markers, they found evidence for a metapopulation structure, showing gene flow within one river basin, but little gene flow between two separated river basins. They also obtained the first mitochondrial DNA data, using a 354 bp mtDNA sequence of the control region, and the results were congruent with the microsatellite analyses. Valenzuela (2001), using eight *P. expansa*-specific microsatellite loci, found significant among-beaches differentiation in four nesting sites within the middle Caqueta river basin, Colombia. Bock *et al.* (2001), investigating *P. expansa* and *P. unifilis* with allozymes, found similar results for *P. expansa* and higher levels of genetic differentiation for *P. unifilis*. Pearse *et al.* (2006) performed a distribution-wide evaluation of population genetic structure and gene flow on *Podocnemis expansa* based on 453 samples from throughout the Amazon region. The mitochondrial DNA sequence and microsatellite results indicated a strong population genetic structure and a general lack of phylogeographic structure. The authors explained this result by a long history of population differentiation in *P. expansa* and the fact that each major tributary of the Amazonian river formed a semi-isolated reproductive population.

Here we report on the first population genetic inventory of *P. lewyana*, using sequences of the mitochondrial cytochrome *b* gene of 109 individuals from various populations located along the four main river basins within its distribution range: Magdalena, Cauca, San Jorge, and Sinú drainages. The Magdalena is the principal river of Colombia. It originates at the bifurcation of the Andean Cordilleras Central and Oriental, and flows northward for some 1,500 km to the Caribbean Sea. The Cauca river is separated from the Magdalena river by the Central Cordillera, and together with the San Jorge river, flows into the Magdalena river in the swampy floodplain of the northern lowlands. During the lower and middle Tertiary, the Magdalena, Catatumbo, and Orinoquian rivers converged and drained to the Maracibo Lake (Schultz, 1949). At that time, the portion of Colombia that corresponds to the current western cordillera and the Caribbean coast was a deep sea. The rising of the eastern cordillera in the superior Miocene separated the Magdalena and Orinoquian drainages (Lundberberg *et al.,* 1986), and also isolated the common Amazonic-Orinoquian fauna. Evidences of such separation are the fossil record of lungfishes, doradid fishes, and the turtle *Podocnemis expansa* in Villa Vieja, Upper Magdalena river (Galvis *et al.,* 1997). Such separation and consequent isolation of the aquatic fauna caused new speciation and dispersion processes in the eastern part of this area. The Sinú river flows

between the eastern and western elevations of the western cordillera, along 482 kilometres through a wide and irregular basin to the Caribbean sea. The Sinú drainage is more recent. At the end of the Pliocene, the sea had retreated from the western cordillera and the Caribbean coast, and the Sinú River already flowed into the Caribbean Sea. The upper part of the Sinú drainage comprises a core of igneous rocks covered by Tertiary sediments, and the majority of the alluvial flatland has Quaternary sediments. The Sinú river forms a separate drainage and although it is located close to the San Jorge river (which belongs to the Magdalena drainage), there is no direct connection between the two rivers (Fig. 2). It is thought that dispersal of *P. lewyana* is occasional and restricted to the rainy season, when some of the temporary water systems are connected with each other. It is, however, unknown if individuals can move from one river basin to the other.

In this paper we address the following two questions: (1) what are the variability levels of cytochrome *b* in populations of *P. lewyana*? And (2) is the genetic variation more homogeneously distributed within a drainage than between drainages?

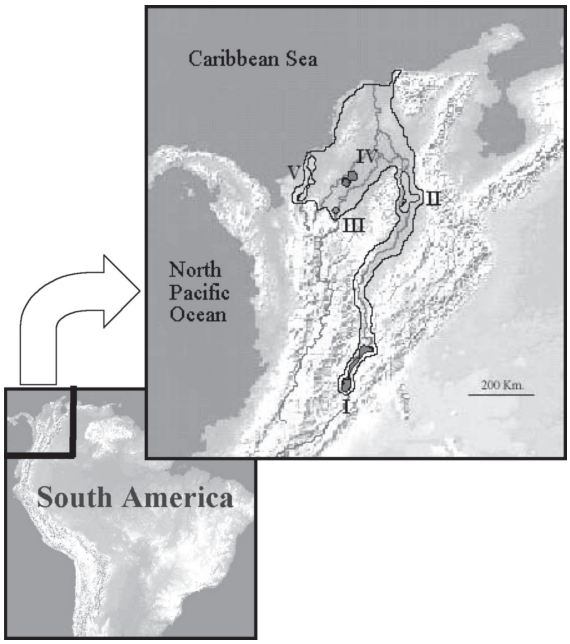

Fig. 2. Map of the five regions sampled over the distribution range of *Podocnemis lewyana.* I: Upper Magdalena River. II: Lower Magdalena River. III: Man River (lower Cauca River). IV: San Jorge River. V: Sinú River. The approximate species' distribution range is indicated by the black line (according to Castaño-Mora and Medem, 2002).

Materials and methods

Blood samples were collected from 109 individuals between November 2004 and April 2005 in 26 localities (grouped in five regions) in the Magdalena, Cauca, San Jorge, and Sinú river basins (Fig. 2). The sample design was such that we collected throughout the entire species distribution range. Techniques like casting nets, snorkeling, and funnel aquatic traps (Feurer, 1980) with green plantain as bait (Gallego-García and Castaño-Mora, in press) were used to catch the turtles. Because part of the fieldwork coincided with Eastertime, many local communities had turtles collected for consumption, which facilitated the gathering of samples. Those turtles were usually caught nearby the communities (Vargas, pers. obs.). We obtained 27 individuals from the upper Magdalena river (region I), 21 individuals from the lower Magdalena river (region II), four individuals from the Cauca river (region III), nine individuals from the San Jorge river (region IV), and 48 individuals from the Sinú river (region V).

A 1 ml syringe with a 6-gauge needle was used to take 50-100 µl of blood from juveniles and 100–200 µl from adults. Blood was taken from the dorsal part of the tail, from the coccigeal dorsal vein. This method does not harm the turtles and it is applicable to individuals of all sizes (Vargas, pers. obs.). After collection, blood samples were immediately preserved in plastic vials containing 1 ml of Queen's lysis buffer (Seutin *et al.,* 1991), stirred until no clots remained, and stored at room temperature. Allowed by a contract of access to genetic resources for a research without commercial interest (Contrato de Acceso a Recursos Genéticos para Investigación sin Interés Comercial), aliquots of all 109 samples were brought to the Institute for Biodiversity and Ecosystem Dynamics (IBED) of the University of Amsterdam, The Netherlands, where the laboratory work was carried out.

Genomic DNA was extracted using a Qiagen DNA-Tissue extraction kit (Qiagen Benelux B.V., Venlo, The Netherlands), following the manufacturer's instructions for extracting DNA from blood. Polymerase Chain Reactions (PCR), used to amplify a fragment of the mitochondrial cytochrome b gene, were performed in a total volume of 25.25 µl containing 17.5 µl H2O, 2.5 µl 10xNH4 superTaq PCR buffer (HT Biotechnology), 1 µl MgCl2 (25 mM), 2 µl dNTPs (1 mM), 1 µl of each primer (10 µM), 0.25 µl of iTaq DNA Polymerase (HT Biotechnology. 5 U/µl), and 1 µl of genomic DNA (approx. 60 ng). Reaction conditions were as follows: initial denaturation of DNA for 90 sec at 94ºC; 34 cycles

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H1: GAGGAGGATTCGCAGTAGACAACGCCACACTCACTCGATTCTTTACATTCCATTTCCTAACCCCATTCATCA
H2: ·····················T·····························································
    TCGCAGGCTTAACAATAATTCACCTCTTATTCCTTCACGAAACAGGATCAAACAACCCCACTGGGTTAAACT
    ·····  ·····  ·····  ·····  ·····  ·····  ·····  ·····  ·····  ·····  ·····
    CAAACACCGATAAAATTCCATTCCATCCATACTTTACATACAAAGACATCCTAGGAATCATGATCCTAATAA
    ·····  ·····  ·····  ·····  ·····  ·····  ·····  ·····  ·····  ·····  ·····
    TATACCTCCTAACCCTATCTATACTTTTACCCAACCTCCTATCAGACCCCGAAAATTTCACACCCGCAAATC
    ·····  ·····  ·····  ·····  ·····  ·····  ·····  ·····  ·····  ·····  ·····
    CCCTTGTCACTCCACCACACATCAAACCAGAGTGATACTTCCTCTTTGCATACGCTATCCTACGATCAATCC
    ·····  ·····  ·····  ·····  ·····  ·····  ·····  ·····  ·····  ·····  ·····
    CAAACAAACTAGGAGGTGTTTTAGCCCTCTTCTTATCAATCGCAATCCTCATCCTTATCCCTACACTTCACAC
    ·····  ·····  ·····  ·····  ·····  ·····  ·····  ·····  ·····  ·····  ·····
    CTCTAAACAACGAACCCTTTCATACCGTCCTATTTCACGGACCCTATTCTGATAC
    ·····  ·····  ·····  ·····  ·····  ·····  ·····  ·····  ·····  ·····  ·····
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Fig. 3. Sequence variation in the 488 bp region of the cytochrome *b* gene; two haplotypes (H1 and H2) were identified in 109 individuals of *Podocnemis lewyana* sampled from five regions. Note the polymorphic site at position twelve.

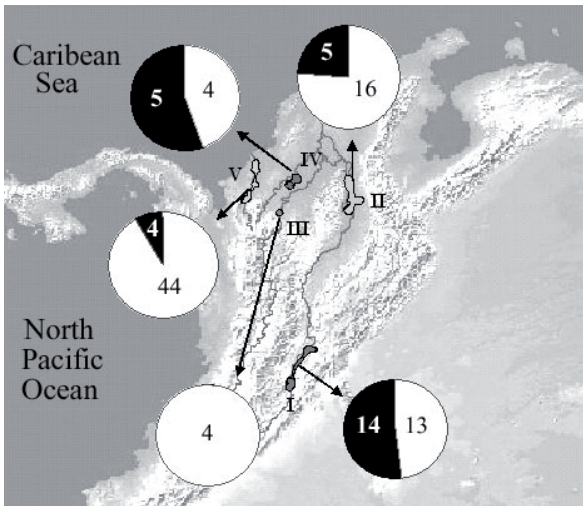

Fig. 4. Number of *Podocnemis lewyana* individuals sharing haplotype H1 (white part of pie diagram) and H2 (black part) of the cytochrome *b* amplified fragment in each of the five regions in Colombia.

of 30 sec denaturing at 94ºC, 45 sec annealing at 45ºC, 30 sec extension at 72ºC; and 5 min final extension at 72ºC. Initially, we used the primers Cytb-C and CBJ10933 (Bossuyt and Milinkovitch, 2000). Based on some of the sequences obtained, we designed one degenerate and one non-degenerate primer,

PodcytbF (5'-GAGGAGGATTCKCAGTAGACA-3') and PodcytbR (5' GTATCAGAATAGGGTCCGTG-3'), using the program OLIGO 4 (MBI, Molecular Biology Insights Inc., Cascade, CO, USA). The PCR conditions to amplify the cytochrome *b* fragment with the newly designed primers were the same as above. The PCR products were then run on a 1.5% low-melt agarose gel, stained with ethidium bromide, and visualized on a "Gel Doc" system. If positive, products were purified using QIAquick spin columns (Qiagen) prior to direct sequencing. The 10.5 µl sequencing reaction included 1 µl of template, 1.5 µl of sequencing buffer, 2 µl of 1 µM primer, 1.8 µl of ready reaction mix (Applied Biosystem), and 4.2 µl of water. The sequence reaction was 33 cycles of 10 sec at 96ºC, 10 sec at 50ºC, and 4 min at 60ºC. Sequence data collection and visualization were performed on an ABI 3100 automated sequencer at the AMC (Academic Medical Center), University of Amsterdam DNA Sequencing Facility. Sequences were deposited in GenBank, accession numbers from EF363561 to EF363669.

Sequences were aligned and edited using Sequence Navigator software (Applied Biosystems). Sequences were translated into amino acid data to check for the presence of stop codons. Sequences were collapsed in haplotypes using the program Collapse version 1.2 (Posada, 1999).

Results

We obtained a total of 488 bp of the cytochrome *b* gene sequence from 109 turtles. No indels or stop codons were detected. In the 488-nucleotide alignment there was only one polymorphic site, a single-nucleotide, silent substitution (transversion) at position 12. The two haplotypes were designated H1 and H2 (Fig. 3).

Haplotype H1 was most common and occurred in 81 specimens out of the 109 sampled (74%; Fig. 4). Different geographic regions had different haplotype frequencies. Region III presented the highest frequency of H1 (100%), possibly because only four individuals (belonging to H1) were collected. Among the regions

where both haplotypes were found, the highest frequency was found in region V (91.7%), followed by region II (76.2%), region IV (55.6%), and region I (48.2%). The regions with the highest frequencies of haplotype H2 were I (51.8%) and IV (44.4%), followed by region II (23.8%) and region V (8.3%)

Discussion

Turtle eggs and turtle meat have been an important food resource for humans for at least the last 20,000 years (Moll and Moll, 2004). Moreover, turtles are highly sought after for traditional medicine in some parts of the world (in particular in Asia) and collected for the pet trade and the food-market (reviewed in Moll and Moll, 2004). These factors, together with habitat alteration and introduced species contribute to the decline of a large number of turtle species in the world (Moll and Moll, 2004). The analysis of the genetic diversity within and among species can help in turtle conservation (Moll and Moll, 2004). Genetic data have been used in turtle conservation to evaluate the genetic variability within and among populations (e.g., Janzen *et al.,* 1997; Souza *et al.,* 2002; Schwartz and Karl, 2006), to recognize the existence of cryptic taxa (e.g., Russello *et al.,* 2005), and to reveal migratory patterns (e.g., in marine turtles see Bowen and Avise, 1996)

Mitochondrial genes have been widely used in turtles for phylogenetic, phylogeographic, and population genetics studies (e.g. Bowen *et al.,* 1993; Barth *et al.,* 2004; Spinks and Shaffer, 2005; Parham *et al.,* 2006). Chiari *et al.* (2005), using the cytochrome *b* gene, recovered three distinct genetic subspecies in the tortoise species *Pyxis arachnoides* in agreement with their geographic separation and plastron differences. Lenk *et al.* (1999) inferred the phylogeography of the European Pond Turtle, *Emys orbicularis,* using sequences of the cytochrome *b* gene and RNA heteroduplex analysis, revealing intraspecific differentiation in 20 different haplotypes with distinct geographical ranges. Fritz *et al.* (2005a), also using cytocrome *b*, investigated the genetic variability of *Emys orbicularis* in Italy, and found a high diversity of mtDNA haplotypes and a complex taxon differentiation in southern Italy. Examination of intra-specific genetic variation in the Western Pond Turtle, *Actinemys marmorata*, has shown low levels of among and within-population divergence (Janzen *et al.,* 1997). However, even in this latter case, cytochrome *b* was able to provide some indication of genetic differentiation between northern and southern populations. Moreover, in the same species Spinks and Shaffer (2005) using fragments of two other mitochondrial genes (control region and ND4) found well defined geographically coherent clades. Considering the slower rate of molecular evolution of the mitochondrial DNA in turtles than in most other taxa (Avise *et a*l., 1992, but see also Seddon, 1998 and Weisrock and Janzen, 2000), even small genetic differentiation in mitochondrial markers can be informative in detecting geographic or historical splits. In fact, Fritz *et al.* (2005b) were able to observe differentiation between populations of *Mauremys leprosa* north and south of the Atlas Mountains even with a maximum of 1% sequence divergence in cytochrome *b*.

In *P. lewyana*, our evaluation of genetic variability based on cytochrome *b* over the distribution range of the species showed low genetic variation within and low differentiation among populations. Only two haplotypes were detected and no clear geographic population substructure was observed. Both haplotypes were found in all but one population, be it in different frequencies. This result suggests that with the cytochrome *b* resolution level, the species can be considered to be genetically uniform. Different river drainages did not show distinctly different patterns, somewhat contrary to what we hypothesized. This result indicates that either high levels of gene flow exist among river basins or that the separation, if any, of the populations is recent (or very slow) so that it did not leave a genetic signal yet. However, considering that the Cauca and San Jorge rivers are tributaries of the Magdalena river, while the Sinú river is not connected with these, the higher frequency of the haplotype H1 observed in the Sinú river could indicate some differentiation. The fixation of haplotype H1 in the Cauca river may be a sampling artefact. Moreover, local people may sometimes move individuals from one place to another (Vargas, pers. obs.), and have probably moved individuals for many years, since *P. lewyana* surely has been used since prehispanic times, as happened with *P. expansa* and *P. unifilis* (Rodriguez *et al.,* 2002). Reintroduction of confiscated specimens by wildlife conservation organizations and local government irrespective of the origin of the animals may also contribute to the mixing of the populations. However, our data should not be interpreted as definitive evidence for managing populations of this species from different environments as a single unit for conservation, especially since morphological, behavioral, and physiological characteristics by which the populations might be adaptively differen-

tiated have not been investigated (Landweber and Dobson, 1999). Further molecular studies using more rapidly evolving markers (e.g., microsatellites) may be able to detect genetic differentiation among *P. lewyana* populations, and to quantify patterns of migration and gene flow between river basins.

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