RESEARCH ARTICLE

Extremely low genetic diversity and weak population differentiation in the endangered Colombian river turtle *Podocnemis lewyana* (Testudines: Podocnemididae)

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Abstract *Podocnemis lewyana* is an endangered endemic river turtle of Colombia. Using ten unlinked polymorphic microsatellite loci and a 691-bp-long DNA fragment corresponding to the more variable portion of the mitochondrial control region, we investigated genetic diversity and population structure throughout its range. Both neutral markers showed extremely low diversity and weak population differentiation. Our data indicate that the genetic history of *P. lewyana* has been impacted by multiple bottlenecks and population expansion since the Pleistocene. The observed differentiation pattern is most likely the result of historically low genetic variation resulting from restricted geographic range and aggravated by recent anthropogenically induced bottlenecks. Based on slight differences in allele frequencies among populations, we suggest that three regions should be treated as demographically independent Management Units in order to preserve maximal genetic diversity: (1) the Upper Magdalena River Basin, (2) the Lower Magdalena + Lower Cauca + San Jorge River Basins, and (3) the Sinú River Basin. Among the Management Units, only low to moderate levels of gene flow were detected; these are largely unidirectional from Management Units 1 and 3 into Management Unit 2.

Resumen *Podocnemis lewyana* es una tortuga endémica y amenazada de Colombia. Usando diez loci polimórficos de microsatélites y un fragmento de 691 pares de bases correspondiente a la porción más variable de la región control mitocondrial, investigamos la diversidad genética y estructura poblacional a través de su rango de distribución. Ambos marcadores neutrales revelaron una diversidad extremadamente baja y débil diferenciación poblacional. Nuestros datos indican que la historia genética de *P. lewyana* ha sido impactada por múltiples cuellos de botella y expansión poblacional desde el Pleistoceno. El patrón de diferenciación observado es más probable el resultado de la variación genética historica baja derivada del rango de distribución restringido, agravada por cuellos de botella recientes resultado de intervención antrópica. Basados en diferencias tenues de frecuencias alélicas entre poblaciones, sugerimos que tres regiones deben ser tratadas como Unidades de Manejo (UM) demográficamente independientes con el fin de preservar al máximo la diversidad genética. (1) Cuenca del alto río Magdalena, (2) Cuencas del bajo río Magdalena + Bajo río Cauca + río San Jorge, y (3) Cuenca del río Sinú. Entre las UM se detectaron niveles bajos a moderados de flujo genético que es principalmente unidireccional de las UM 1 y 3 a la UM 2.

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Introduction

The turtle family Podocnemididae, known since the Cretaceous, comprises many extinct and eight extant large freshwater species from South America and Madagascar. Podocnemididae and its sister group, the Afrotropical family Pelomedusidae, are the last survivors of a formerly diverse radiation of turtles, the Pelomedusoides (Gaffney et al. 2006). The genus *Podocnemis* contains six extant South American species (*P. erythrocephala*, *P. expansa*, *P. lewyana*, *P. sextuberculata*, *P. unifilis*, *P. vogli*; Fritz and Havaš 2007). *P. expansa* is listed in the threat category "Lower Risk/Conservation Dependent" by the IUCN Red List of Threatened Species (IUCN 2010); *P. erythrocephala*, *P. sextuberculata* and *P. unifilis* are treated as "Vulnerable", and *P. lewyana* is classified as "Endangered". Among the many chelonians under extreme pressure from human exploitation (Klemens 2000), *Podocnemis* species deserve particular attention as

conservation targets because they represent ancient lineages (Vargas-Ramírez et al. 2008; Gaffney et al. 2011).

Podocnemis lewyana is an endangered river turtle, with a distinctly smaller range than most other podocnemidids. It is a medium-sized to large, strictly aquatic species with maximum shell lengths of 32 cm and 46 cm in males and females, respectively. The species is confined to the drainage systems of the Sinú, San Jorge, Cauca, and Magdalena Rivers of Colombia (Fig. 1), and the only podocnemidid northwest of the Andes (Páez et al. 2009). As one of the three chelonian species endemic to Colombia (besides *P. lewyana*: *Kinosternon dunni*, *Mesoclemmys dahli*; Fritz and Havaš 2007), the country has a special responsibility to conserve *P. lewyana*. Phylogenetically, *P. lewyana* is sister to the Amazonian blackwater species *P. erythrocephala* (Vargas-Ramírez et al. 2008).

Populations of *P. lewyana* are dwindling throughout its distribution range. The Magdalena River Basin is one of the most important Colombian regions in terms of
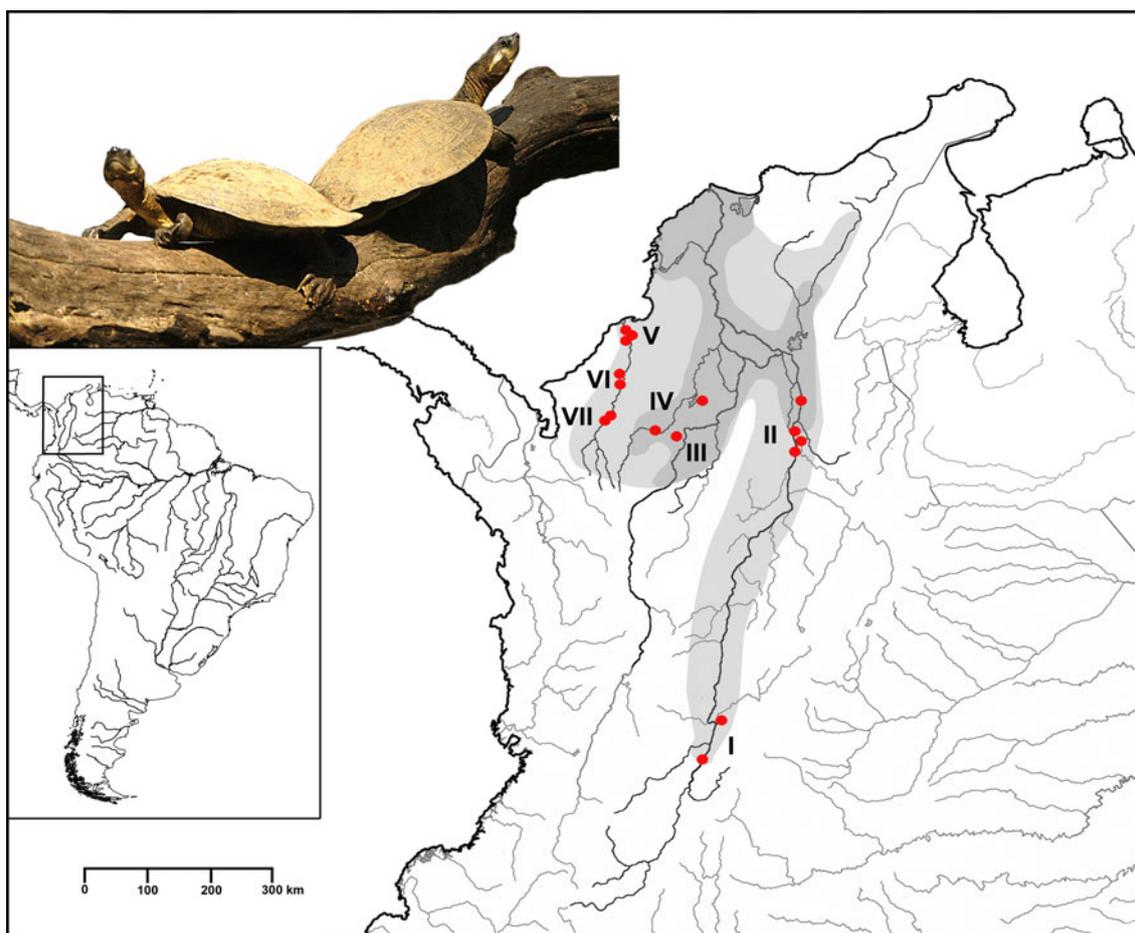
Fig. 1 Distribution of *P. lewyana* in Colombia (light grey) and sampling sites (*dots*). Roman numerals refer to collecting regions: I: Upper Magdalena, II: Lower Magdalena, III: Man River, Lower Cauca, IV: San Jorge River, V: Lower Sinú River, VI: Middle Sinú River, VII: Upper Sinú River. Mompos Depression (Depresión Momposina; see "Discussion" section) shown in *dark grey*

development and economy. Traditionally, its waters have produced more than 60% of fish consumed in the country, but fish populations collapsed since the 1970s (Galvis and Mojica 2007). The same factors, habitat destruction and pollution, non-sustainable overexploitation and hydrological changes due to dams, contribute to the decline of *P. lewyana* (Gallego-García 2004; Vargas-Ramírez et al. 2006, 2007; Gallego-García and Castaño-Mora 2008; Páez et al. 2009). This situation, combined with the species' limited distribution range, makes *P. lewyana* the most threatened of all *Podocnemis* species.

Little is known about the genetic variation of *P. lewyana*. Vargas-Ramírez et al. (2007) studied a 488-bp-long fragment of the mitochondrial cytochrome *b* gene in 109 individuals from five regions in the Magdalena and Sinú River Basins and found only two haplotypes, differing by only one substitution. By contrast, in two other *Podocnemis* species high levels of genetic diversity were discovered with mtDNA and microsatellite data (Pearse et al. 2006; Escalona et al. 2009). In the present study, we use rapidly evolving nuclear and mitochondrial markers [microsatellites, variable part of the control region (CR)] and a range-wide sampling to assess population genetic structure, bottlenecks, gene flow, and population expansion in *P. lewyana*. We aim at providing a solid foundation for effective management measures under the growing pressure by the increasing human population. The necessary prerequisite for such efforts is knowledge of the population structure that allows the identification of Management Units sensu Moritz (1994), i.e., of populations with significant divergence of allele frequencies at nuclear or mitochondrial loci. *P. lewyana* lives within a

biodiversity hotspot (Myers et al. 2000) and shares its range with a plethora of other taxa, among them a number of endemic fish, amphibian and bird species (Ruiz-Carranza et al. 1996; Acosta-Galvis 2000; Buitrago-Suárez and Burr 2007; Galvis and Mojica 2007; Maldonado-Ocampo et al. 2008; Salaman et al. 2009). Since chelonians are charismatic animals, *P. lewyana* has the potential to become a flagship species for the conservation of its entire ecosystem and all co-distributed species. Our study is embedded in a long-lasting conservation and management program for the species supported by the National University of Colombia and an NGO (Biodiversa Colombia).

Materials and methods

Sampling and laboratory procedures

Between November 2004 and April 2008, blood samples were collected from wild populations in seven regions (four river basins), representing the entire distribution range of *P. lewyana* (Fig. 1; Table 1). The turtles were hand-captured by snorkelling, using traditional fishing nets called "atarrayas", or obtained from local fishermen. Fifty to one-hundred μl of blood were taken from the coccygeal vein of juveniles, and 100–200 μl from that of adults. Blood samples were immediately preserved in plastic vials containing 1 ml of Queen's lysis buffer (Seutin et al. 1991) and stirred until no clots remained. Frozen blood samples are permanently housed in the Instituto de Ciencias Naturales, Universidad Nacional de Colombia, Bogotá;

Table 1 Genetic parameters inferred from microsatellite and CR data for *P. lewyana*

| Region | Basin | Localities | Microsatellites (nDNA) | | | | | | | Control region (mtDNA) | | | |
			N	A_{ob}	A_R	H_O	H_E	PA	PAf	N	h	π	Haplotypes
I	Upper Magdalena	Prado, Melgar, Girardot	49	4.20	1.93	0.271	0.261	9	0.01–0.08	33	0.365	0.00055	A, B, C
II	Lower Magdalena	Rio Lebrija, Caño Grande, Cienaga de Doña Maria	31	4.50	2.35	0.358	0.350	8	0.02–0.03	26	0.520	0.00098	A, D, E, F, G, H, I
III	Lower Cauca	Caucasia	4	2.50	2.50	0.425	0.337	2	0.12	4	0.833	0.00145	A, E, J
IV	San Jorge	Ayapel, Marralú	9	3.90	2.75	0.422	0.418	4	0.05	6	0.333	0.00048	A, J
V	Upper Sinú	Tierra Alta, Valencia	22	2.80	1.90	0.250	0.259	2	0.04–0.09				
VI	Middle Sinú	Monteria, Jaraquiel	13	2.70	2.10	0.300	0.300	0	–	50	0.040	0.00006	A, K
VII	Lower Sinú	Caño Viejo, Cotocá, Lorica	19	3.00	2.08	0.321	0.310	1	0.05				
	Total		147	–	–	–	–	26		119	–	–	–
	Mean/ overall		–	6.90	2.24	0.335	0.319	–		–	0.292	0.00006	–

Sinú River regions were lumped together for mitochondrial data

N sample size; A_{ob} mean observed number of alleles per locus; A_R allelic richness; H_O observed heterozygosity; H_E unbiased expected heterozygosity; PA number of private alleles per basin; PAf private alleles frequency range; h haplotype diversity; π nucleotide diversity

subsamples were imported to Germany using the proper CITES permits. Genomic DNA was extracted using a Qiagen DNA blood extraction kit (Qiagen Benelux B.V., Venlo, The Netherlands), following the manufacturer's instructions. Polymerase Chain Reaction (PCR) with fluorescently labelled primers (6-FAM, HEX, PET, NED, Biomers, Ulm, Germany) was used to individually amplify 13 microsatellites originally designed for *P. unifilis* (Fantin et al. 2007) and five additional ones for *P. expansa* (Sites et al. 1999; Valenzuela 2000). Seven markers for *P. unifilis* (Puni1B2, Puni1B10, Puni1B11, Puni1F10, Puni2A9, Puni2C11, Puni2D9, Fantin et al. 2007) and three for *P. expansa* (Sat62, Sat128, Sites et al. 1999; PE519, Valenzuela 2000) proved to be useful for *P. lewyana* by successful cross-amplification and appropriate levels of polymorphism; the presence of microsatellite repeats was confirmed by sequencing on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Two markers each were combined in duplex PCRs. Thermocycling conditions for the first three duplex PCRs were as follows (primer pairs Sat128 + Puni2C11, Puni1B11 + Puni1F10, Puni1B10 + Puni1B2): Initial denaturing for 1 min at 94°C, 25 cycles with denaturing 50 s at 94°C, annealing 50 s at 60°C, and primer extension for 1 min at 72°C, followed by 20 cycles with denaturing 40 s at 92°C, annealing 50 s at 50°C, and primer extension for 40 s at 68°C and a final elongation of 20 min at 72°C. For the primer pairs Puni2A9 + Puni2D9 and Sat62 + PE519, the annealing temperature was 64°C in the first set of cycles; the other conditions were the same.

Since Vargas-Ramírez et al. (2007) found virtually no variation in the mitochondrial cytochrome *b* gene of *P. lewyana*, the more rapidly evolving CR was chosen as mitochondrial marker. For amplifying and sequencing 691 bp of the more variable portion of the CR, the primer pair LewDLFor1/LewDLRev1 (5′-TAAGTCTAGGAGAGTTT ACGCG-3′/5′-GGTAGATCATCTCCAATTACGG-3′) was designed. PCR was performed in a 50 μl volume (PCR buffer, Bioron, Ludwigshafen, Germany, or 50 mM KCl, 1.5 mM MgCl$_2$, and 10 mM Tris–HCl, 0.5% Triton X-100, pH 8.5) containing 1 unit of *Taq* DNA polymerase (Bioron), 10 pmol dNTPs (Fermentas, St. Leon-Roth, Germany) and 5 or 10 pmol of each primer. Thermocycling conditions were as follows: Initial denaturing for 3 min at 94°C, 5 cycles with denaturing 30 s at 94°C, annealing 30 s at 50°C, and primer extension for 60 s at 72°C, followed by 35 cycles with denaturing 30 s at 94°C, annealing 30 s at 48°C, and primer extension for 60 s at 72°C and a final elongation of 5 min at 72°C. PCR products were purified using ExoSAP-IT (USB Corporation, Cleveland, OH, USA) and sequenced directly on both strands on an ABI 3130xl Genetic Analyzer. GenBank accession numbers of the obtained haplotypes are HQ704378-HQ704388.

Data analyses

Microsatellites

Genetic diversity

To assess genetic diversity within river basins, Hardy–Weinberg equilibrium and pairwise linkage disequilibrium were calculated for all loci using the exact test implemented in ARLEQUIN 3.11 (Excoffier et al. 2005). Significance levels were estimated using a Markov chain permutation with 10,000 steps and 1,000 dememorization steps. Observed heterozygosity (H_O) and Nei's unbiased expected heterozygosity (H_E) under Hardy–Weinberg equilibrium were also calculated in ARLEQUIN. Allelic richness (A_R), observed allelic diversity (A_{ob}) and the number of alleles per population and locus were calculated using FSTAT 2.9.3.2 (Goudet 2001). GENALEX 6.1 (Peakall and Smouse 2005) was used to reveal the presence and number of private alleles and MICRO-CHECKER 2.2.3 (van Oosterhout et al. 2004) to test for the presence of null alleles. All significance levels for tests involving multiple comparisons were Bonferroni-corrected (Rice 1989).

Population differentiation

Population structure was inferred with two independent Bayesian clustering methods: (i) a spatially explicit method using exact geographic coordinates as prior information and searching for partitions which are similar with respect to their allele frequencies (BAPS 5.3; Corander et al. 2003) and (ii) a spatially non-explicit method searching for partitions corresponding to populations in Hardy–Weinberg and linkage equilibrium (STRUCTURE 2.3.2, without sampling location information; Pritchard et al. 2000; Hubisz et al. 2009). Their parallel application is advantageous for corroborating the results because the packages employ different computational approaches and BAPS is thought to better identify clusters when F_{ST} values are small (Latch et al. 2006; François and Durand 2010).

Using STRUCTURE, posterior probabilities were calculated for $K = 1$ to $K = n + 3$ with $n = 7$, corresponding to the number of distinct collection regions. The number of clusters (K) was assessed using an admixture scenario with allele frequencies correlated. The admixture model assumes recent or current gene flow, so that individuals can have ancestors from more than one population. Correlated allele frequencies are expected to occur in populations with common origin. The burn-in was set to 2×10^4 and the number of further MCMC runs to 8×10^4; calculations were repeated 20 times for each K. Convergence of likelihood values was reached after the burn-in (values were

not decreasing or increasing). For estimation of K, posterior probabilities [highest $\ln P(D)$] and the ΔK method (Evanno et al. 2005) were used. Barplots were produced to visualize the obtained results for clusters and individual admixture. In BAPS, the number of genetic clusters was estimated by performing five independent runs for different maximum Ks (from $K = n$ to $K = n + 10$), where n was again the number of distinct collection regions (7). Using 500 simulations from posterior allele frequencies, the probability of genetic admixture between each cluster was assessed and individual barplots were obtained.

In order to estimate how much individuals, a priori defined populations and Bayesian clusters contributed to the global genetic variation, a hierarchical AMOVA (Excoffier et al. 1992) as implemented in ARLEQUIN was performed. Pairwise F_{ST} values were also calculated using ARLEQUIN.

Bottlenecks

Three methods to detect signatures of population declines were applied, in an attempt to position such population disturbances in a temporal framework. The first method, implemented in BOTTLENECK 1.2.02 tests across all loci for a significant observed heterozygosity excess relative to heterozygosity expected for the number of observed loci (Cornuet and Luikart 1996; Piry et al. 1999). For each sample, the per-locus deviation from expected heterozygosity under a mutation model is calculated by the program, and averaged across all loci. For microsatellites the two-phase mutation model (TPM) was chosen, because it is more suitable for microsatellite data than the strict one-step stepwise mutation model (SMM; Piry et al. 1999). The test parameters were: 95% single-step mutation, 5% multiple step mutation, and the variance among multiple steps was set to 12 as suggested by Piry et al. (1999). Statistical significance was determined using a Wilcoxon sign-rank test (Luikart and Cornuet 1998).

The second method, also implemented in BOTTLE-NECK 1.2.02, evaluates whether the expected distribution of allele frequency classes conformed to expectations of mutation-drift equilibrium. When the rare allele frequency class (0.00–0.10) is less common than other classes, it is likely that the population suffered a bottleneck. This test is qualitative rather than statistical, but is useful in detecting recent bottlenecks (less than a few dozen generations; Luikart et al. 1998).

The third method, the M-ratio test (Garza and Williamson 2001), uses a different attribute of the behaviour of microsatellite loci in population bottlenecks. It calculates the test statistic $M = k/r$, where k is the number of alleles and r the overall range in fragment sizes. A declining population will have a smaller M-ratio than a stable one

because in a small population, k is expected to decrease faster than r due to genetic drift causing the loss of rare alleles. To simulate the expected M values, the program CRITICAL_M (Garza and Williamson 2001) was used. The first test parameters were: $\theta = 1$, proportion of one-step mutations $p_s = 0.88$, and average size of non-one-step mutation $\Delta_g = 2.8$ (as suggested by Garza and Williamson 2001; Kuo and Janzen 2004). To assess the effects of changes in parameter values for the consistency of the results, additional runs were performed using θ values ranging from 0.1 to 10, p_s values ranging from 0.8 to 0.99, and Δ_g values ranging from 2.5 to 6 (Kuo and Janzen 2004). The critical value M_c, below which bottlenecks are inferred, was determined using the software M-CRIT (Garza and Williamson 2001; http://swfsc.noaa.gov/textblock.aspx?Division=FED&id=3298).

The parallel application of all three methods allows exploration of bottleneck effects at a relative timescale (Spear et al. 2006). This concept is based on the fact that the M-ratio recovers much more slowly than heterozygosity excess and rare allele distribution (Garza and Williamson 2001). Thus, if a bottleneck signature results from all three methods, a recent or current population decline can be inferred. By contrast, populations showing only a low M-ratio, but not significant heterozygosity excess or changes in allelic distribution, most probably underwent a historical decline in size.

Detection of migrants

In order to assess asymmetrical migration across populations, a Bayesian multilocus genotyping procedure based on Markov chain Monte Carlo (MCMC) methods as implemented in BAYESASS (Wilson and Rannala 2003) was used. In contrast to the classical indirect estimators of gene flow, this method does not require populations to be in Hardy–Weinberg equilibrium. For the first run default settings were used. Subsequent runs incorporated different Δ values for allele frequency, migration rate, and inbreeding to ensure that proposed changes between chains at the end of the run were between 40 and 60% of the total chain length (Wilson and Rannala 2003). When the run achieved the acceptable proposed changes, four subsequent runs were performed using different random seed numbers in order to ensure consistency among runs. The values from the four runs were averaged. The program ran for 2×10^7 iterations with the first 10^6 iterations discarded as burn-in. Samples were collected every 2,000 iterations to infer the posterior probability distributions of the migration proportions and individual immigrant ancestries revealed by a population assignment test (Rannala and Mountain 1997).

Mitochondrial DNA

mtDNA sequences of 119 individuals were generated, containing the more variable portion of the mitochondrial CR. In 28 individuals the CR fragment could not be amplified. Sequences were edited and aligned using CHROMAS 1.51 (http://www.technelysium.com.au/chromas.html) and BIOEDIT 7.0.5.2 (Hall 1999). Using TCS 1.21 (Clement et al. 2000), all sequences were then collapsed in haplotypes, resulting in 11 haplotypes. Evolutionary relationships among haplotypes were inferred using the statistical parsimony algorithm of TCS. Haplotype diversity (h) and nucleotide sequence diversity (π) were calculated in DNASP 5.0 (Librado and Rozas 2009) range-wide and per region to evaluate the genetic signature of possible historical demographic changes. Haplotype frequencies, nucleotide composition, numbers of transitions and transversions, the number of polymorphic sites and the genetic differentiation among sampled river basins and pairwise F_{ST} values were calculated with ARLEQUIN. Using the population structure revealed by STRUCTURE and BAPS, a hierarchical AMOVA was also performed. Furthermore, to check for population expansion a mismatch distribution test was run in ARLEQUIN and the expansion parameters τ, θ_1 and θ_0 were calculated. ARLEQUIN estimates parameters of a sudden demographic (or spatial) expansion using a generalized least-square approach (Schneider and Excoffier 1999). The sum of square deviations (SSD) between the observed data and the data from the simulated expansion model were used as a test statistic; a significant P value rejects the fit of the data to the expansion model (Rosenbaum et al. 2007). Tajima's D and Fu's F_S values were also calculated in ARLEQUIN; significantly negative D and F_S values suggest historical population growth (Tajima 1989; Fu 1997). Pairwise mismatch distribution graphs were produced in DNASP 5.0. These methods are expected to either identify past demographic events such as population expansion or to detect when demographic historical information has disappeared due to the influence of recent population bottlenecks (Weber et al. 2004; Johnson et al. 2007).

Isolation-by-distance

To assess the possible influence of isolation-by-distance (IBD) patterns on population structuring, Mantel tests as implemented by IBD (Bohonak 2002) were used for microsatellite and mitochondrial data. Pairwise population values of $F_{ST}/(1 - F_{ST})$ were compared against geographical distances corresponding to the shortest river route between each pair of populations (Fetzner and Crandall 2003).

Results

Microsatellites

Genetic diversity

None of the studied loci differed significantly from Hardy–Weinberg expectations. In addition, no significant linkage disequilibrium between any pair of loci was detected; neither for any river basin nor across basins after Bonferroni correction. Subsequently, microsatellite data were used to analyze diversity in all seven collecting regions (Table 1). The number of alleles for each locus ranged from two (Puni1B2, Puni1B10, Puni2A9) to 21 (PE519); Puni2D9 showed 12 alleles and the others less than 10 alleles (Table S1). From a total of 69 alleles, 26 private ones were found at low frequency. The highest number of private alleles was found in the Upper Magdalena and Lower Magdalena (Table 1). Allelic richness (A_R) per population ranged from 1.90 to 2.75 (mean: 2.24) and the observed allelic diversity (A_{ob}) was slightly higher (2.50–4.50; overall: 6.90; Table 1). Expected heterozygosity was low across river basins and ranged from 0.259 to 0.418 (mean: 0.319); the lowest value was observed in Upper Sinú and the highest in San Jorge. Null alleles were not detected.

Population differentiation

To reveal population structuring among the 147 turtles, two different Bayesian algorithms were used as implemented in the software packages STRUCTURE (searching for genetic populations in Hardy–Weinberg and linkage equilibrium) and BAPS (searching for genetic populations showing characteristic allele frequencies). Their parallel application is advisable because they employ different algorithms and BAPS is thought to better identify clusters when F_{ST} values are small (Latch et al. 2006; François and Durand 2010), which is the case in our data set (Table S2).

STRUCTURE found three distinct units ($K = 3$, revealed by the highest mean posterior probability and ΔK method; Table S3). One cluster corresponded to the Upper Magdalena (I); another one to the Lower Magdalena (II) + Lower Cauca (III) + San Jorge (IV); and the third one to the Sinú River (V + VI + VII). This structuring was independently confirmed by BAPS ($K = 3$; confidence of $P = 99\%$). These units, especially cluster 2, showed a high degree of admixture (Fig. 2).

A hierarchical AMOVA revealed that 7.18% of the total variation occurred among these three major Bayesian clusters ($F_{CT} = 0.0718$; $P = 0.00001$). Among the a priori defined populations within these clusters only 1.12% occurred ($F_{SC} = 0.01203$; $P = 0.102$). The vast majority

Table 2 Results of the tests for past bottlenecks in *P. lewyana*

Site	Heterozygosity excess	Allele frequency	M-ratio test	
			M_c	M-ratio
Lower Magdalena	0.0005*	–	0.80 (10)	0.59
Lower Cauca	0.05*	–	0.76 (7)	0.42
San Jorge	0.001*	–	0.78 (10)	0.58
Upper Sinú	0.04*	–	0.80 (8)	0.48
Middle Sinú	0.0001*	–	0.89 (10)	0.58
Lower Sinú	0.005*	–	0.80 (9)	0.50
Cluster 1 (Upper Magdalena)	0.055	Normal	0.81 (9)	0.44
Cluster 2 (Lower Magdalena, Lower Cauca, San Jorge)	0.0005*	Shifted	0.81 (10)	0.47
Cluster 3 (Sinú)	0.0068*	Shifted	0.81 (10)	0.46

Heterozygosity excess values bearing an asterisk are statistically significant. In the *M*-ratio test, values below M_c indicate bottlenecks. In brackets, number of loci contributing to the *M*-ratio test

of the total genetic variation (91.7%) was found within a priori defined populations, i.e., on the individual level ($F_{ST} = 0.08199$; $P = 0.002$). However, Mantel tests of an IBD analysis revealed no correlation between genetic divergence (pairwise F_{ST}) and geographic distances ($Z = 2.53$, $r = 0.31$, $P = 0.256$), so that it can be concluded that, despite the weak differentiation, the observed divergence is not due to IBD but to population differentiation.

Bottlenecks

Evidence for bottlenecks was detected for all seven collecting regions and for all three clusters suggested by STRUCTURE and BAPS (Table 2). The test for heterozygosity excess was non-significant only for the Upper

Magdalena River, although the value of 0.055 was very close to the significance limit (the Upper Magdalena River corresponds to the distinct cluster 1 of STRUCTURE and BAPS). The allele frequency test was run only for the three population clusters of STRUCTURE and BAPS, because it cannot be reliably applied to sample sizes below 30 individuals (Cornuet and Luikart 1996). A modal shift in allele frequency classes as expected from a recent bottleneck was found for the clusters 2 and 3, but not for cluster 1 (Upper Magdalena). For all runs of the *M*-ratio test using different parameters, consistent results were obtained. The *M*-ratios were below the M_c thresholds for all sampling regions and all population clusters, indicating bottlenecks.

A direct comparison of the results of all three bottleneck analyses provides evidence for an old bottleneck in the Upper Magdalena River that predates the events observed
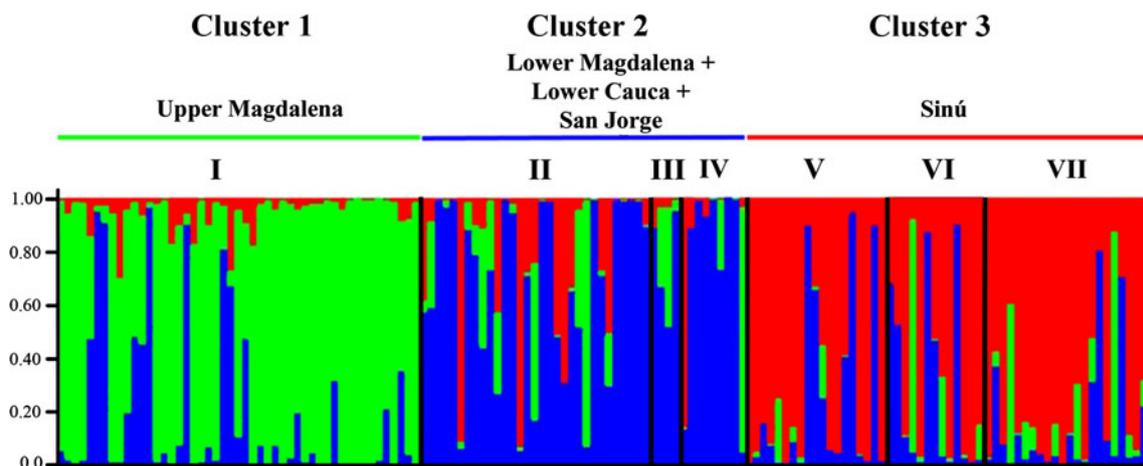
Fig. 2 Barplots from STRUCTURE suggesting three suggesting three clusters ($K = 3$) based on 10 unlinked polymorphic microsatellite loci (shown is the run with the highest mean posterior probability)

Table 3 Gene flow estimate for each cluster pair in a metapopulation of *P. lewyana*

| Cluster pair	Gene flow (*m*)
1–2	0.099 (0.014–0.204)
2–1	0.008 (0–0.035)
1–3	0.007 (0–0.030)
3–1	0.017 (0.000–0.064)
2–3	0.008 (0–0.040)
3–2	0.172 (0.071–0.275)

Estimates of gene flow represent the mean migration rate and 95% confidence interval (in brackets) for a pair of clusters (subpopulations)

in all other a priori defined populations and Bayesian clusters. This conclusion is derived from the low *M*-ratio in combination with the non-significant test for heterozygosity excess and the normal allelic distribution in the Upper Magdalena.

Detection of migrants

When the BAYESASS analyses of migration were run with the seven a priori defined populations corresponding to the collecting regions, inconsistencies were detected between different runs among populations with no (or extremely low) differentiation. It has been suggested that the estimates of short-term migration rates and individual migrant ancestries may have a low accuracy for weakly differentiated populations (Wilson and Rannala 2003). Therefore, we present here only the estimates for the three units identified by STRUCTURE and BAPS. Using these clusters, consistent results were obtained (Table 3) that suggested asymmetric migration, with a much stronger influx from cluster 1 (Upper Magdalena) to cluster 2 (Lower Magdalena + Lower Cauca + San Jorge) than vice versa (Fig. 3). A similar situation occurred with respect to clusters 2 and 3 (Sinú). In this case a much stronger immigration was suggested from cluster 3 to cluster 2 than in the opposite direction (Fig. 3). For cluster 1, total emigration was estimated as 0.157, total immigration 0.025, and net emigration 0.132. For cluster 2, total emigration was 0.016, total immigration 0.271, and net emigration −0.0254. For cluster 3, total emigration was 0.189, total immigration 0.014, and net emigration 0.175. To enable an easy evaluation of data quality for migration rates, the 95% confidence intervals are reported along with the means for these values (Table 3), as suggested by Wilson and Rannala (2003).

The relatively isolated status of the Upper Magdalena (cluster 1) and Sinú (cluster 3) was also suggested by population assignment tests using BAYESASS (Table S4). The majority of turtles of these two clusters were identified as residents (no migrants) and only low percentages as migrants or migrant's offspring. In contrast, only 16.32% of the individuals of cluster 2 were identified as residents and 28.01% as
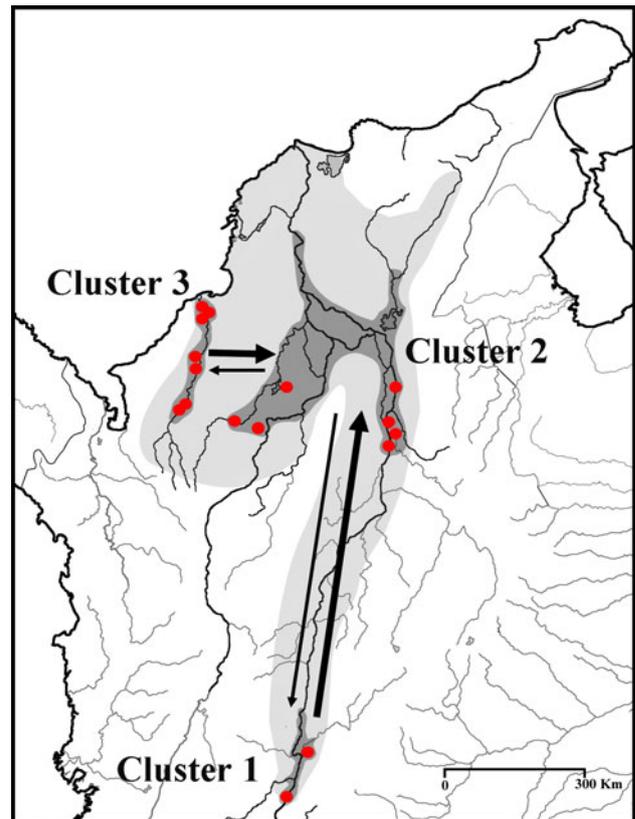
Fig. 3 Gene flow pattern among populations of *P. lewyana*. *Arrows* indicate direction of gene flow from Cluster 1 (Upper Magdalena River) and Cluster 3 (Sinú River Basin) into Cluster 2 (Lower Magdalena + San Jorge + Lower Cauca Rivers). Relative amount of directional gene flow is represented by the thickness of each *arrow*

migrants from the other two clusters and 55.67% as migrant's offspring, matching the high amount of admixture for this cluster as suggested by STRUCTURE (see above).

Mitochondrial DNA

Based on the comparison of a 691-bp-long fragment of the mitochondrial CR of 119 *P. lewyana*, 11 distinct haplotypes were identified (Table S5), differing in 11 polymorphic sites (nine transitions and two transversions; Table S6). The most frequent haplotype A was found in all populations. In three regions (Upper Magdalena, Lower Magdalena, Sinú) private haplotypes occurred (Tables 1, S5). The haplotype network showed a star-like structure (Fig. S1) and most of the haplotypes were distinct from the most frequent and ancestral haplotype A by one mutational step; the only exception was haplotype H, which differed by two mutational steps. The overall haplotype diversity (*h*) was 0.292 and nucleotide diversity (*π*) was 0.00006. Individual values for each of the seven study regions are summarized in Table 1.

In order to explore demographic events, Tajima's *D* and Fu's *F*$_S$ values were computed and mismatch distribution

Table 4 Comparison of genetic parameters inferred from microsatellite and CR data of *P. lewyana* (bold) and other continental chelonian species

Taxon	Region	Marker									Source
		Microsatellite nDNA					mtDNA				
		N	Loci	H_O	H_E	A_R	N	Fragment	h	π	
Astrochelys radiata	Madagascar	323	13	0.76	0.80	8.90	–	–	–	–	Rioux Paquette et al. (2009)
Emydoidea blandingii	Nova Scotia, Canada	129	5	0.55	0.55	8.00	–	–	–	–	Mockford et al. (2005)
Emys orbicularis	Western, central and southern Europe	382	8	0.63	0.72	5.15	–	–	–	–	Pedall et al. (2011)
Emys trinacris	Sicily, Italy	31	8	0.55	0.68	4.86	–	–	–	–	Pedall et al. (2011)
Gopherus berlandieri	Texas, USA	138	8	0.47	0.52	5.21	–	–	–	–	Fujii and Forstner (2010)
Graptemys geographica	Ontario, Canada	109	5	0.65	0.63	4.15	–	–	–	–	Bennett et al. (2010)
Malaclemys terrapin	Gulf and Atlantic coasts, USA	320	6	0.76	0.79	7.44	–	–	–	–	Hauswaldt and Glenn (2005)
Podocnemis unifilis	Amazon and Orinoco Basins	312	5	0.69	0.70	7.25	–	–	–	–	Escalona et al. (2009)
Terrapene ornata	Illinois, Nebraska, USA	74	11	0.57	0.72	9.27	–	–	–	–	Kuo and Janzen (2004)
Chelonoidis nigra vandenburghi	Galápagos Islands, Ecuador	162	10	0.60	0.68	8.7	135	CR	0.52	0.0044	Beheregaray et al. (2003)
Emys orbicularis	Spain	261	7	0.72	0.71	5.27	261	CR, ND4	0.81	0.00187	Velo-Antón et al. (2008)
Macrochelys temminckii	Mississippi River, USA	195	7	0.36	0.37	2.38	195	CR, tRNA-Pro	0.50	0.001	Echelle et al. (2010)
Podocnemis expansa	Amazon and Orinoco Basins	453	9	–	0.75	6.4	293	CR	0.65	0.00256	Pearse et al. (2006)
Podocnemis lewyana	**Colombia**	**147**	**10**	**0.33**	**0.32**	**2.24**	**119**	**CR**	**0.29**	**0.00006**	**This study**

N sample size; H_O observed heterozygosity; H_E unbiased expected heterozygosity; A_R allelic richness; h haplotype diversity; π nucleotide diversity

analyses performed (i) for all regions as given in Table 1, except that localities at the Sinú River were grouped together, and (ii) for all river basins lumped together (Table S7). The distribution of pairwise nucleotide differences was consistent with expectations of a unimodal distribution indicating population expansion (Fig. S2; Table S7). The Tajima's D, and Fu's F_S values were negative, and in the Lower Magdalena, Sinú, and all regions lumped together D and F_S were statistically significantly different from zero, again suggesting population expansion.

According to a hierarchical AMOVA, 7.05% of the total variation occurred among the three major Bayesian clusters ($F_{CT} = 0.07059$; $P = 0.01$), and only 0.43% of the total variation was found among populations within these clusters ($F_{SC} = 0.00429$; $P = 0.4511$). The majority of global genetic variation (92.52%) was found on the individual level within the a priori defined populations ($F_{ST} = 0.07458$; $P = 0.007$). Since the Mantel tests revealed no correlation between genetic

and geographic distances ($Z = 281.03$, $r = 0.34$, $P = 0.184$), the observed genetic structure cannot be interpreted as the result of IBD and it has to be concluded that it reflects weak, but discernable population differentiation.

Discussion

Population structure in the face of low genetic variability and gene flow

The levels of heterozygosity, allelic richness, and haplotype and nucleotide diversities indicate that *P. lewyana* is a species with extremely low genetic diversity. This is underlined by a comparison with other chelonian species (Table 4). The distinctly lower values of *P. lewyana* strongly contrast with most of the other species, some of which are or were heavily exploited and experienced sharp

population declines (*Astrochelys radiata*, *Chelonoidis nigra*, *Macrochelys temminckii*, *P. expansa*, *P. unifilis*).

Despite this low diversity, the two Bayesian clustering algorithms independently unravelled evidence for three distinct genetic clusters within *P. lewyana*, corresponding to (1) The Upper Magdalena River Basin, (2) the Lower Magdalena + Lower Cauca + San Jorge River Basins, and (3) the Sinú River Basin. Yet, the hierarchical AMO-VAs (for microsatellites and mtDNA) indicate that most of the global variation (approx. 92% for both markers) occurs on the individual level, but that some 7% of the variation corresponds to differences among the three clusters. Thus, our data provide evidence for a discernable, albeit weak population structure in *P. lewyana*.

Along the Magdalena River, *P. lewyana* is rare and the species has apparently experienced marked declines in most sites where it still exists (Restrepo et al. 2008). Although several intermediate localities between the Upper and Lower Magdalena River were visited during the course of our investigation (where the species is known to occur), the few turtles observed were spotted only in wide and deep parts of the river, where it was not possible to catch them. Even baited funnel traps were unsuccessful, suggesting the species is very rare there. The genetic characterization of intermediate populations which connect cluster 1 with cluster 2 must remain therefore pending, and we cannot exclude the possibility that part of the observed population differentiation corresponds to anthropogenically induced subdivision by local extirpation.

Historical demography

Our analyses indicate that the genetic history of *P. lewyana* has been influenced by multiple bottlenecks throughout the distribution range. The results of the *M*-ratio test in combination with the analyses of heterozygosity excess and allele frequency shifts suggest an old bottleneck in the Upper Magdalena region. The same analyses indicate, however, at least one secondary, younger decline in genetic diversity in the other areas of the distribution range. Indeed, the signal for the *M*-ratio test can persist for hundreds of generations, depending on the rate of mutation and population turnover (Garza and Williamson 2001). *P. lewyana* as a large-sized turtle has a long life-expectancy, which suggests together with the low mutation rate in turtles (Avise et al. 1992; FitzSimmons 1998) that the signal for a past bottleneck may be preserved for a very long time.

Like the northern hemisphere, South America has been severely impacted by Pleistocene glaciations (Clapperton 1993). The effects of the Last Glacial Maximum (LGM) on the hydrological and habitat conditions in the Magdalena River Basin, previously influenced by the rain shadow of the uplift of the western Cordillera and Serranía of Perijá,

were surely considerable, corresponding to a marked reduction in the amount of water present in the drainage (Galvis et al. 1997), and resulting in pronounced changes of the aquatic and terrestrial ecosystems. Furthermore, in the Holocene at least four additional dry periods (5000, 2000, 700, and 500 years ago) have affected the Magdalena River Basin (Livingstone and van der Hammen 1978; Archila-M. 1993). It seems reasonable to suppose that such historical events acted in concert with subsequent human impact. The old bottleneck in the Upper Magdalena could result from Pleistocene reductions in population size while younger events, including anthropogenic pressure, are likely to be reflected by the evidence for the more recent decline.

Large parts of the distribution range of *P. lewyana* were transformed by pre-Hispanic cultures, suggesting that anthropogenic impact can last for centuries. The Mompos Depression, today with low human population density, was densely inhabited in pre-Hispanic times. The Zenú and their ancestors built a large and complex network of canals extending over more than 500,000 hectares of swamp (Plazas et al. 2005), and there is archaeological evidence for the species' utilization by indigenous peoples (Puerto Hormiga: from 5,000 to 4,500 years ago, Mompos Depression: 2,500–750 years ago, Valle de Santiago: 1,600–1,000 years ago; Archila-M. 1993). This suggests that the extreme level of exploitation of turtle meat and eggs as known today (Vargas-Ramírez et al. 2006) may have occurred for many centuries, and a similar scenario recently has been proposed for a Central American river turtle (*Dermatemys mawii*; González-Porter et al. 2011). Such intensive historical harvesting, along with the current exploitation and habitat destruction, may have caused the genetic signal for recent population decline. In any case, the small distribution range of *P. lewyana* surely has contributed to the observed low diversity.

Compared to the other study regions, genetic differentiation is most pronounced in the Upper Magdalena River. Among others, two private mitochondrial haplotypes (one of them identified in six individuals) occur there and the highest number of private alleles. This suggests that the Upper Magdalena may have harboured a population of *P. lewyana* that was isolated during past dry periods.

Much of the distribution range of *P. lewyana* corresponds to the Mompos Depression (Depresión Momposina, Fig. 1), the largest swamp region of Colombia with 24,650 km^2 (Anonymus 2002). It is located between the Caribbean lowland and the Serranías of Ayapel, San Lucas and Perijá. This interior delta, where the waters from the Magdalena, César, San Jorge and Cauca Rivers converge, experiences periodic inundations and remains flooded from April until November (Plazas et al. 2005). Its abundance of water suggests that the Mompos Depression served as an important refuge during past dry periods, whereas the

relatively high degree of admixture argues also for a role as genetic hub for the distinct populations.

However, our data indicate that, apart from bottleneck events, population expansion is also likely to have shaped the current population structure. Evidence for population expansion is mainly provided by the unimodal distribution of pairwise differences between mtDNA haplotypes (mismatch distribution; Fig. S2). By contrast, bottlenecks are expected to show multimodality in mismatch distributions (Rogers and Harpending 1992; Weber et al. 2004; Johnson et al. 2007). Thus, the results of the mismatch distribution analysis suggest a signature of population expansion, and this is further supported by our observation of negative Tajima's D and Fu's F_S values and the star-like haplotype network topology (Fig. S1; Table S7). We interpret this finding as evidence for a recovery of individual numbers after historical bottlenecks.

Conservation implications

Our study revealed for *P. lewyana* a clear population structure, but overall very low levels of genetic diversity and allelic richness. This has key implications for conservation. The present decline of *P. lewyana* throughout its distribution range is known to be caused by overexploitation and habitat destruction (Vargas-Ramírez et al. 2006; Gallego-García and Castaño-Mora 2008; Restrepo et al. 2008; Páez et al. 2009). This will certainly aggravate the low genetic diversity, so that strategies are needed to counteract further loss of diversity. The population structure of *P. lewyana* implies that conservation efforts cannot focus only on one part of the distribution range. For preserving the present genetic diversity, at least three independent Management Units (Moritz 1994) need to be considered, i.e., (1) the Upper Magdalena River Basin, (2) the Lower Magdalena + Lower Cauca + San Jorge River Basins, and (3) the Sinú River Basin.

Reserves should be established in each of these three regions and in the region between the Upper and Lower Magdalena River sections because it corresponds to an important gateway for genetic exchange. In general, migration routes along the river corridors and temporal body waters that connect river basins need to be maintained for ensuring gene flow (e.g., seasonal water connections between the Sinú River Basin and Mompos Depression populations).

Of outstanding importance in this context is to accept the challenge and responsibility of assuring the long-term survival of *P. lewyana*. We encourage the Colombian government through the Environmental Ministry to promote a dialogue among stakeholders (research institutes, regional environmental corporations, governmental and non-governmental organizations, zoos, land owners, human communities, etc.) to collaborate on efforts for in-situ and ex-situ preservation of the species. In order to effectively reduce the consumption of eggs and adults, or at least to achieve a sustainable use, community-based local education programs need to be implemented. Previous efforts were successful on a local scale (Vargas-Ramírez et al. 2006, 2009; for the Upper Magdalena River: Vargas-Ramírez 2007; for the Sinú River: Gallego-García 2006). Now it is time to learn from these experiences and to expand them over all distribution regions. The future of *P. lewyana*, and of all of its many co-distributed species, is in our hands.

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